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IONIZATION CONSTANTS OF BENZOTHIADIAZINES

by

© URSULA G. HENNIG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

PHARMACEUTICAL CHEMISTRY

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EDMONTON, ALBERTA

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "IONIZATION CONSTANTS OF BENZOTHIADIAZINES" submitted by Ursula G. Hennig in partial fulfilment of the requirements for the degree of Master of Science in Pharmaceutical Chemistry.

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DATE October 2, 1979

Dedicated to my parents,

who provided unfailing emotional support

to just one more slave of 'The System'

ABSTRACT

The diuretic potency and duration of action of the benzothiadiazines has been ascribed to either the lipid solubility of the drugs or to their competitive inhibition of the renal active transport of p-aminohippurate. Both of these factors appear to be influenced by the ionization constants of the benzothiadiazines. A limited aqueous solubility and the instability of these agents in alkaline media have prevented the determination of their overlapping acidic ionization constants by conventional methods.

One of the most suitable methods for determining the acidity constants of sparingly soluble drugs, the solubility variation with pH technique, does not take into account instability problems. The preparation of reference decomposition products and the TLC analysis of sample solutions at various time intervals, during the solubility studies of methyclothiazide and bendroflumethiazide, indicated that decomposition takes place during the agitation and equilibration periods. This decomposition in buffers of pH 8 and higher was confirmed with the acidified p-dimethylaminobenzaldehyde test for primary aromatic amines.

In an attempt to analyze all of the selected drugs by the same technique, the thermodynamic values of the dissociation constants of a series of benzothiadiazines and diazoxide were determined, wherever possible, by the ultraviolet spectrophotometric method. The superimposed UV absorption spectra which were obtained during the investigation were also used to theorize the order of deprotonation of the

two acidic hydrogens. This UV spectral information of the benzothiadiazines and diazoxide, together with the NMR spectral studies and the effect of substitution at the 3-position, suggests that the hydrogen at the 2-position is the more acidic.

The ionization constants of the benzothiadiazines appear to be of considerable importance to the pharmacological activity of these agents. It is apparent that the diuretic potency of the benzothiadiazines is directly related to the lipid solubility of these drugs. Thus, a lower dosage is required in order to achieve a maximum effect and the increased lipid solubility also extends their duration of action. The lipid solubility increases or decreases, relatively, with the decreased or increased acidity of each of the benzothiadiazines.

Diazoxide does not possess diuretic activity and this is probably due to the absence of an exocyclic sulfonamido group. Structure-activity relationships involve the exocyclic sulfonamido hydrogens and the effect of substitution at the 3-position of the benzothiadiazines indicates that the hydrogen at the 2-position is influenced by the nature of this substituent.

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE SURVEY	7
Conductimetry	8
Aqueous Potentiometric Titrimetry	9
Semiaqueous Potentiometric Titrimetry	11
The Extrapolation Technique	13
Ultraviolet Spectrophotometry	16
Fluorimetry	24
Nuclear Magnetic Resonance Spectrometry	25
Solubility Variation with pH	30
Partition-Distribution Studies	37
Dissociation Constants of Benzothiadiazines	38
STATEMENT OF THE PROBLEM	43
EXPERIMENTAL	46
Apparatus and Reagents	47
Buffers	47
Reference Standards	49
Procedures	52
A. Preparation of Reference Decomposition Products	52
B. Detection of Benzothiadiazine Decomposition by Thin Layer Chromatography	54
C. pKa Determination by Solubility Variation with pH	56

Page

D. UV Absorption Spectra of Benzothiadiazines and Diazoxide 60

E. Determination of Benzothiadiazine Acidity Constants by UV Spectrophotometry 62

F. Determination of Deprotonation Order. 64

DISCUSSION AND RESULTS. 68

The Decomposition of Benzothiadiazines 69

Solubility Studies 78

Methods of Determining Benzothiadiazine Acidity Constants. . 83

UV Absorption Spectra of Benzothiadiazines and Diazoxide . . 86

UV Spectral Data. 95

Acid-Base Chemistry. 135

Data for Methyclothiazide, Polythiazide and Diazoxide 139

Overlapping Ionization Constants 147

Absorbance-pH Data. 162

Computer Input and Output Data. 182

NMR in Determining the Order of Deprotonation. 201

SUMMARY AND CONCLUSIONS 212

BIBLIOGRAPHY. 217

APPENDIX. 223

LIST OF TABLES

Table	Description	Page
1	Literature Values of Benzothiadiazine Dissociation Constants	42
2	Buffers Transparent to Ultraviolet Light	48
3	UV Absorbance Readings of Methyclothiazide at Selected pH Values	80
4	Absorbance Changes for Methyclothiazide Relative to Equilibration-Agitation Periods	82
5	Data for the Determination of the Acidic pKa Value of Methyclothiazide	140
6	Determination of the Acidic Dissociation Constant of Methyclothiazide	141
7	Data for the Determination of the Acidic pKa Value of Polythiazide	142
8	Determination of the Acidic Dissociation Constant of Polythiazide	143
9	Data for the Determination of the Acidic pKa Value of Diazoxide	144
10	Determination of the Acidic Dissociation Constant of Diazoxide	145
11	Data for the Determination of the Overlapping pKa Values of Hydrochlorothiazide	164
12	Data for the Determination of the Overlapping pKa Values of Hydroflumethiazide	166
13	Data for the Determination of the Overlapping pKa Values of Trichloromethiazide	168
14	Data for the Determination of the Overlapping pKa Values of Althiazide	170
15	Data for the Determination of the Overlapping pKa Values of Flumethiazide	172
16	Data for the Determination of the Overlapping pKa Values of Chlorothiazide	174

Table	Description	Page
17	Data for the Determination of the Overlapping pKa Values of Bendroflumethiazide.	176
18	Data for the Determination of the Overlapping pKa Values of Cyclothiazide.	178
19	Data for the Determination of the Overlapping pKa Values of Cyclopenthiazide	180
20	Computer Input and Output Data for Hydrochlorothiazide.	183
21	Computer Input and Output Data for Hydroflumethiazide	186
22	Computer Input and Output Data for Trichloromethiazide.	189
23	Computer Input and Output Data for Althiazide.	191
24	Computer Input and Output Data for Flumethiazide . . .	193
25	Computer Input and Output Data for Chlorothiazide. . .	194
26	Manual Calculations for Resolution of the Overlapping pKa Values of Chlorothiazide and Bendroflumethiazide.	195
27	Computer Input and Output Data for Bendroflumethiazide.	196
28	Computer Input and Output Data for Cyclothiazide . . .	197
29	Computer Input and Output Data for Cyclopenthiazide	198
30	Acidity Constants of Various Benzothiadiazines and Diazoxide as Determined by Ultraviolet Spectrophotometry.	199
31	Comparisons of the Acidity of Benzothiadiazines and Diazoxide Obtained by Various Techniques	200
32	NMR Assignments for Methyclothiazide and Hydrochlorothiazide Before the Addition of Titrant . .	208
33	Chemical Shift Data for the Titration of Hydrochlorothiazide and Methyclothiazide	210

Table	Description	Page
34	Percent Ionization of the Acidic Functional Groups of Hydrochlorothiazide at Each Incremental Addition of Titrant	211
35	Computer Program Reprinted from "The Determination of Ionization Constants" (20)	224
36	Computer Program for the Resolution of Overlapping pKa Values.	229

LIST OF FIGURES

Figure	Description	Page
1	Calibration Curve for Methyclothiazide in Solubility Studies Determined at the Isobestic Point	79
2	Absorption Spectra of Diazoxide 2 x 10^{-5} M at Various pH Values	98
3	Absorption Spectra of Diazoxide 2 x 10^{-5} M at Various pH Values	99
4	Absorption Spectra of Diazoxide 2 x 10^{-5} M at Various pH Values	100
5	Absorption Spectra of Chlorothiazide 2 x 10^{-5} M at Various pH Values	101
6	Absorption Spectra of Chlorothiazide 2 x 10^{-5} M at Various pH Values	102
7	Absorption Spectra of Chlorothiazide 2 x 10^{-5} M at Various pH Values	103
8	Absorption Spectra of Flumethiazide 2 x 10^{-5} M at Various pH Values	104
9	Absorption Spectra of Flumethiazide 2 x 10^{-5} M at Various pH Values	105
10	Absorption Spectra of Flumethiazide 2 x 10^{-5} M at Various pH Values	106
11	Absorption Spectra of Benzthiazide 2 x 10^{-5} M at Various pH Values	107
12	Absorption Spectra of Benzthiazide 2 x 10^{-5} M at Various pH Values	108
13	Absorption Spectra of Benzthiazide 2 x 10^{-5} M at Various pH Values	109
14	Absorption Spectra of Hydrochlorothiazide 2 x 10^{-5} M at Various pH Values.	110
15	Absorption Spectra of Hydrochlorothiazide 2 x 10^{-5} M at Various pH Values.	111

Figure	Description	Page
16	Absorption Spectra of Hydroflumethiazide 2 x 10^{-5} M at Various pH Values	112
17	Absorption Spectra of Hydroflumethiazide 2 x 10^{-5} M at Various pH Values	113
18	Absorption Spectra of Hydroflumethiazide 2 x 10^{-5} M at Various pH Values	114
19	Absorption Spectra of Bendroflumethiazide 2 x 10^{-5} M at Various pH Values	115
20	Absorption Spectra of Bendroflumethiazide 2 x 10^{-5} M at Various pH Values	116
21	Absorption Spectra of Bendroflumethiazide 2 x 10^{-5} M at Various pH Values	117
22	Absorption Spectra of Bendroflumethiazide 2 x 10^{-5} M at Various pH Values	118
23	Absorption Spectra of Cyclothiazide 2 x 10^{-5} M at Various pH Values	119
24	Absorption Spectra of Cyclothiazide 2 x 10^{-5} M at Various pH Values	120
25	Absorption Spectra of Cyclothiazide 2 x 10^{-5} M at Various pH Values	121
26	Absorption Spectra of Cyclopenthiazide 2 x 10^{-5} M at Various pH Values	122
27	Absorption Spectra of Cyclopenthiazide 2 x 10^{-5} M at Various pH Values	123
28	Absorption Spectra of Althiazide 2 x 10^{-5} M at Various pH Values	124
29	Absorption Spectra of Althiazide 2 x 10^{-5} M at Various pH Values	125
30	Absorption Spectra of Althiazide 2 x 10^{-5} M at Various pH Values	126
31	Absorption Spectra of Trichloromethiazide 2 x 10^{-5} M at Various pH Values	127

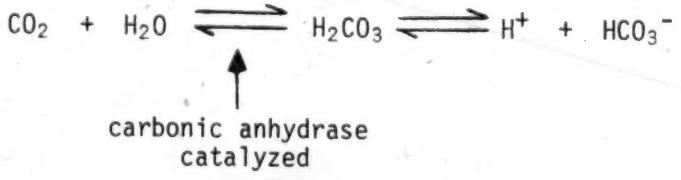
Figure	Description	Page
32	Absorption Spectra of Trichloromethiazide 2×10^{-5} M at Various pH Values	128
33	Absorption Spectra of Methyclothiazide 2×10^{-5} M at Various pH Values	129
34	Absorption Spectra of Methychlothiazide 2×10^{-5} M at Various pH Values	130
35	Absorption Spectra of Methyclothiazide 2×10^{-5} M at Various pH Values	131
36	Absorption Spectra of Polythiazide 2×10^{-5} M at Various pH Values	132
37	Absorption Spectra of Polythiazide 2×10^{-5} M at Various pH Values	133
38	Absorption Spectra of Polythiazide 2×10^{-5} M at Various pH Values	134
39	Graphical Representation of Absorbance-pH Data of Methyclothiazide, Polythiazide and Diazoxide	146
40	Graphical Representation of Absorbance-pH Data of Hydrochlorothiazide 2×10^{-5} M	165
41	Graphical Representation of Absorbance-pH Data of Hydroflumethiazide 2×10^{-5} M	167
42	Graphical Representation of Absorbance-pH Data of Trichloromethiazide 2×10^{-5} M	169
43	Graphical Representation of Absorbance-pH Data of Althiazide 2×10^{-5} M	171
44	Graphical Representation of Absorbance-pH Data of Flumethiazide 2×10^{-5} M	173
45	Graphical Representation of Absorbance-pH Data of Chlorothiazide 2×10^{-5} M	175
46	Graphical Representation of Absorbance-pH Data of Bendroflumethiazide 2×10^{-5} M	177
47	Graphical Representation of Absorbance-pH Data of Cyclothiazide 2×10^{-5} M	179

Figure	Description	Page
48	Graphical Representation of Absorbance-pH Data of Cyclopenthiazide 2×10^{-5} M	181
49	Graphical Representation of Chemical Shift Data for the Titration of Hydrochlorothiazide 0.03 M . . .	209

INTRODUCTION

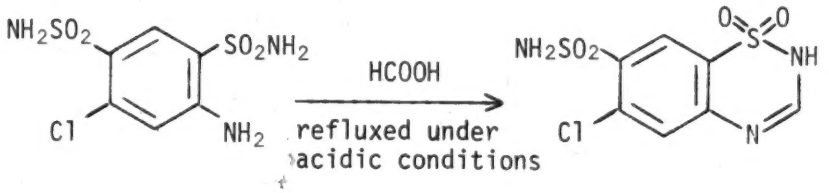
Structure-activity relationship studies of aromatic sulfonamides began when patients on sulfonamide chemotherapy developed an interesting side effect, namely diuresis and an alkaline urine. Sulfanilamide itself exhibited a moderate degree of carbonic anhydrase inhibitory action and subsequent investigations revealed that enzyme inhibition and diuretic effect increased with the more acidic sulfonamides. This correlation between acidity and biological activity inspired the advent of potent carbonic anhydrase inhibitors such as acetazolamide, however, these compounds exerted their diuretic action by promoting sodium bicarbonate excretion and this eventually caused an electrolyte imbalance, leading to metabolic acidosis (1-3).

The enzyme carbonic anhydrase is responsible for keeping bicarbonate in equilibrium with metabolically produced carbon dioxide and also for acidifying the urine (1).



The enzyme also regulates bicarbonate reabsorption in the proximal tubule of the kidney (3). In the presence of a carbonic anhydrase inhibitor the secretion of hydrogen ions derived from carbonic acid is suppressed and the normal exchange for sodium ions is, consequently, decreased. The excess sodium ions combine with bicarbonate ions and the subsequent excretion of sodium bicarbonate is accompanied by an increased volume of water and a substantial quantity of potassium ions.

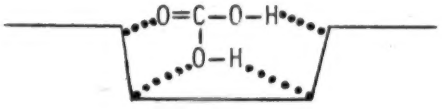
An ideal diuretic should promote the excretion of both sodium and chloride ions, as did the otherwise toxic mercurials. Introducing a second sulfonamido group on the aromatic ring of benzenesulfonamides enhanced chloride excretion while retaining carbonic anhydrase inhibitory action. Formic acid mediated ring closure of one particular benzenedisulfonamide, 4-amino-6-chloro-1,3-benzenedisulfonamide (DSA), produced chlorothiazide (4).



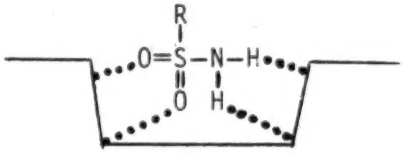
DSA chlorothiazide

Chlorothiazide was found to be mildly active as a carbonic anhydrase inhibitor but it produced marked chloride excretion. Although orally effective, its large dosage requirement prompted structural modifications that eventually led to the large class of salidiuretic compounds known as benzothiadiazines. Further investigation of their pharmacological activity (5-10), together with the clinical success of chlorothiazide (1-3, 9-12), resulted in a series of mild to moderate carbonic anhydrase inhibitors. Many of these benzothiadiazines were orally effective at doses that were one hundred times lower than the dose required for chlorothiazide, and ultimately, these drugs replaced the mercurial agents as diuretics in medical practice.

Carbonic anhydrase inhibitory activity is attributed to the free sulfonamido group on the aromatic ring and substitution of one or both of the sulfonamido hydrogens results in the loss of activity, with respect to carbonic anhydrase inhibition. A schematic representation of the proposed interaction of the drug with the enzyme is as follows (9):



The active site of carbonic anhydrase saturated with carbonic acid.



The active site of carbonic anhydrase saturated with a sulfonamide.

The major diuretic effect of the benzothiadiazines, however, is due to the inhibition of the reabsorption of sodium and chloride ions in the distal segment of the kidney tubule and this direct effect on renal tubular transport is independent of carbonic anhydrase inactivation (1).

Drugs of the benzothiadiazine class are subdivided into two categories (13): thiazides, which are 1,2,4-benzothiadiazine-1,1-dioxides, and hydrothiazides, which are 3,4-dihydro-1,2,4-benzothiadiazine-1,1-dioxides. Thiazides and hydrothiazides are low ceiling diuretics (1,3) and during therapy a low plateau or low ceiling of activity is reached, therefore, their mild onset of action and the 6-10 hours duration of action cannot be enhanced by additional or increased doses (3). This property, together with the low toxicity

potential of the benzothiadiazines, provides a therapeutic advantage in diuretic maintenance therapy. Benzothiadiazines are now also routinely prescribed in the treatment of hypertension (1,2,14) and congestive heart failure (1,2,15).

The mechanism of antihypertensive action of the benzothiadiazine class of drugs has not been completely elucidated but the presently accepted theory defines a dual mechanism of antihypertensive effect (1,14). An initial decrease in blood pressure, due to extracellular fluid volume and plasma volume depletion, is followed by a direct vasodilator action resulting in decreased peripheral vascular resistance.

The diuretic potency and duration of action of the benzothiadiazines has been ascribed to two main factors. firstly, the lipid solubility of the drugs, which appears to determine the distribution and consequently the concentration of drug in subcellular fractions (16-18), and secondly, to a competitive inhibition of the renal active transport of p-aminohippurate (19). Both of these factors appear to be influenced by the ionization constants of the benzothiadiazines. The procedures employed to determine these constants have often been adaptations of recognized methods for determining the dissociation constants of organic substances. The adjustments in methodology are necessary to accommodate some of the troublesome physical and chemical properties of the benzothiadiazines, which are their limited aqueous solubility and their instability in alkaline media.

The primary difficulty arising in the determination of benzothiadiazine dissociation constants is the very low aqueous solubility of

these compounds. In view of this fact, and in recognition of the voluminous available literature dealing with methodology, the literature survey will emphasize methods that are designed for and, therefore, are particularly amenable to the determination of ionization constants for sparingly soluble substances.

LITERATURE SURVEY

Generally, any physical property which exhibits a constant variation with protonation is potentially suitable for determining acidity constants, and conductance, optical, and precise emf measurements are considered to be the most reliable for this purpose. The review of selected aspects of previously well-documented methodology (20-22) is intended to examine both the merits and the limitations of each method. A particular emphasis will be placed on the methods utilizing the conventional aqueous pH scale, and acidity constants related to extensions of this pH scale have not been reviewed.

The possible methods of determining ionization constants which have been outlined and discussed include the methods of choice (aqueous potentiometric titrimetry and ultraviolet spectrophotometry), acceptable methods (fluorimetry, nuclear magnetic resonance spectrometry, solubility variation with pH, and distribution studies), and methods that are controversial but are, nevertheless, widely employed (semiaqueous potentiometric titrimetry and the extrapolation technique). In addition, literature methodology relating specifically to the benzothiadiazines has also been reviewed.

Conductimetry

The oldest method of determining acidity constants is conductimetry (20,21). The measurement of the equivalent conductance of an aqueous standard solution is compared to the measured or calculated limited conductances of successively more dilute sample solutions and these comparisons yield the degree of ionization from which the acidity constant is calculated.

Disadvantages of the conductimetric method are many. One of the major drawbacks is the acute sensitivity to quite small temperature variations and accurate temperature control is imperative since a 2% increase in conductivity occurs per degree temperature rise. In addition, small amounts of ionic impurities interfere with conductivity readings and the high conductivity of salts prevents suitable measurements at a constant ionic strength. Furthermore, dissolved carbon dioxide presents another problem as carbonic acid suppresses the ionization of weak acids (those acids with a pKa value of above 6.5). The method also requires lengthy and tedious calculations and it is not easily adapted to the determination of overlapping ionization constants.

Aqueous Potentiometric Titrimetry

Once the accuracy of potentiometric titrimetry became evident, it rapidly replaced conductimetry as the method of choice in determining acidity constants (20). Fewer calculations are required and the technique is more rapid and versatile, as well as accurate and practical.

A readily available and easily operable instrument, the pH meter, fitted with a glass-calomel electrode combination is suitable for the pH range of 2-11 (20-22). The potential of the glass electrode varies linearly with pH and standardization is achieved with commercially available buffers. Following the standardization, a closed titration vessel is kept at a constant temperature, the sample solution is stirred, preferably with a gentle stream of nitrogen (20,22), and titrated with acid or base. Potassium hydroxide is the titrant of choice for acids

since a high ratio of potassium to hydrogen ions does not affect the accuracy of the glass electrode as readily as a high ratio of sodium to hydrogen ions (20). Hydrochloric acid is the most commonly used titrant for bases.

For successful aqueous titration the compounds under investigation should be soluble in water so as to provide, at least, 0.01 M concentrations. Albert and Serjeant (20) insist that the glass electrode is only accurate over the 0.005-0.05 M concentration range but routine analysis should provide reasonably accurate measurements in more dilute solutions. In fact, a precision pH apparatus does not increase the accuracy of pH measurements involving sparingly soluble substances (20,22) and a pH meter calibrated in 0.02 pH units is quite adequate. For concentrations of less than 0.01 M, sufficient equilibration time must be provided and, as the concentration being titrated decreases, the equilibration time must be increased (21).

Measuring the pH at the point of half neutralization of the compound is only sufficient for determining apparent acidity constants, whereas for thermodynamic values an indication of activities and precision is necessary (20-22). For precise results, 7-10 points on the titration curve, in the range of the pKa±1 unit, should be chosen for the final calculations (20,22). Corrections for hydrolysis effects must be made for readings below pH 4 and above pH 10 for 0.01 M concentrations and for measurements below pH 5 and above pH 9 for 0.001 M concentrations (20). These corrections are made using hydrogen ion activity in the acidic pH range and hydroxyl ion activity in the basic pH range.

The treatment of the data involves relating the stoichiometric concentrations of the molecular and the ionized species to the sample solution pH and this degree of ionization-pH relationship yields the negative logarithm of the acidity constant, denoted as the pKa. The aqueous potentiometric technique can be extended to encompass the calculation of the overlapping acidity constants of a compound (those ionizations that are separated by less than three pKa units) and the procedure requires the titration of two ionizable groups.

Concentrations of less than 0.001 M are very difficult to titrate accurately and duplication of the results is often unattainable. The problem is intensified by the fact that some compounds are slowly soluble in water or they may only be sparingly water soluble and, hence, the sample concentration is too low to permit a meaningful and accurate titration. Precipitation from the solution during the titration procedure is also a possible occurrence with sparingly soluble compounds. Sometimes it is advantageous to titrate a sparingly soluble acid, or one that decomposes when it is heated to aid its dissolution, in the form of its sodium salt. The sodium salt is then titrated with hydrochloric acid but this back titration is only successful if the salt is considerably more soluble than the acid itself (20).

Semiaqueous Potentiometric Titrimetry

Due to the fact that solubility problems frequently preclude the titration of aqueous sample solutions, and absorption spectrophotometry is a time-consuming means of determining acidity constants,

semiaqueous potentiometric titrations became popular. This titrimetric method has also gained favor with compounds that are not suitable for spectrophotometric analysis. In brief, the method is a potentiometric titration, as previously described, differing only in the solvent composition of the sample solution. Rather than an aqueous solution, a predetermined ratio of water to miscible organic solvent is employed, the nonaqueous component being selected with respect to the solubility requirements of the substance under investigation.

The technique provides relative pKa values and it is suitable for comparing the acidities of structurally similar compounds (20) but it is not precise enough for the comparison of dissimilar compounds that have been titrated in the same solvent system or for the comparison of structurally similar compounds that have been titrated in different aqueous-organic solvent systems. In such solvent combinations, the pH readings that are obtained should not, by definition, be related to the aqueous pH scale. Bates et al. (23) have devised an operational pH scale in methanol-water and in ethanol-water solvent systems and this allows for some comparison of the acidities of compounds that are not structurally similar. Further work has brought about reference buffer solutions that are ideal for pH measurements in 50% methanol (24).

The accuracy of the glass electrode in nonaqueous or in semiaqueous solvents has been questioned since the dehydration of the pH sensitive tip is a possibility and because the predictability of the liquid-junction potential is also affected by these solvent systems (21). Gutbezahl and Grünwald (25) have suggested strict standardization and equilibration procedures in aqueous buffers but, on the contrary, Bates

et al. (23) have observed an unimpaired electrode response at solvent compositions of below 90% wt. alcohol. Further 'proof' of glass electrode accuracy is stated by Bacarella et al. (26) in their studies of dioxane-water and methanol-water solvent systems. These investigators claim that any discrepancies between the pH readings which are obtained in slightly varying aqueous-organic solvent compositions are of no more significance than the experimental error involved in the measurements and, furthermore, they suggest that the glass electrode be stored, equilibrated, and standardized in a solvent of exactly the same composition as that required by the experiment.

The Extrapolation Technique

In spite of much opposing information, the temptation to relate the acidities obtained by semiaqueous potentiometric titrations to aqueous systems and the conventional pH scale has not been suppressed. The extrapolation technique, which is now extensively employed, is an extension of the semiaqueous titration method. The most common procedure is the titration of sample solutions of varying solvent composition, followed by the extrapolation to 100% water content and solutions containing 20-60% of organic solvent are most commonly employed in preventing the precipitation of a sparingly soluble compound during titration. Extrapolation becomes difficult and undependable when compounds are too insoluble to provide pKa values in solutions of less than 20% organic solvent content (20). Successful extrapolation requires sample solutions of 5 and 10% organic solvent content in order

to interpret the 'hockey-stick' type of curves described by Albert and Serjeant (20), Saunders and Srivastava (27), and Cavill et al. (28).

A modification of the extrapolation technique replaces solvent composition with the dielectric constant of the particular solvent system involved. Pring (29) and Edmonson and Goyan (30), to mention only a few, investigated this possibility and extrapolated to the dielectric constant of water but this procedure, in effect, has not altered the theoretical aspects and disadvantages previously described.

To further extend the extrapolation technique, Chatten and Harris (31), in addition to measuring the pH at the point of half neutralization in varying solvent compositions, titrated varying concentrations of the therapeutic agents they were investigating. This method allowed them to extrapolate to infinite dilution and the technique provided accurate apparent pKa values for selected sympathomimetic amines and phenothiazines. Chatten et al. (32) used the same method for methaqualone but obtained an anomalous pKa value, as was confirmed by the ultraviolet spectrophotometric method of Patel et al. (33) and Zalipsky et al. (34), as well as the fluorimetric technique of Schulman et al. (35).

Successful determinations of the pKa values for numerous pharmaceutical substances have been achieved by using the extrapolation technique. Marshall (36) determined the acidity constants of various antihistamines in ethanol-water systems, Stockton and Johnson (37) found the pKa of sulfathiazole in alcohol-water mixtures, Chatten and Harris (31) studied phenothiazines and sympathomimetic amines in methanol-water solvents, and Garrett (38) assigned apparent dissociation

constants to the functional groups of tetracyclines by using dimethylformamide-water solvent systems.

Peeters (39) has proposed a method that is an alternative to the conventional, time-consuming extrapolation technique. A single, continuous titration can be performed by preparing an initial solution of known solvent composition which is then titrated with an appropriate titrant. During the titration the solvent composition will continuously change and the exact composition at each step of the titration can be calculated. Since the addition of the aqueous titrant will increase the water content of the sample solution, the titration, in itself, will extrapolate to 0% organic solvent.

Albert and Serjeant (20) have attributed the anomalies observed in semiaqueous solvent systems to the lipophilicity of certain substances. The molecular species, which is lipophilic, is surrounded by a cage of low-dielectric-constant solvent molecules while the ionic species, which is hydrophilic, is surrounded by water molecules. As a result, this theory suggests that for a highly lipid soluble compound the ionic forms, as well as the molecular form, will be much more soluble in the organic solvent than in the aqueous component. This possibility would impede the use of solvent compositions containing less than 30 or 40% of organic solvent, the ultimate result being a partitioning effect (21).

Cookson (21) has suggested two major complicating factors in the use of semiaqueous solvents in the determination of acidity constants. Firstly, the complexities created by the different solvating power of the two solvent components, and secondly, the extra acidic and basic species that are contributed to the solution by the organic solvent.

The importance of the solvent-structural aspects in hydrogen bonded solvent mixtures may indicate that the organic solvent which most closely resembles water is the most suitable for semiaqueous titrimetry and the extrapolation technique. Ethanol and methanol fit these criteria in ultraviolet spectrophotometric studies but their effect in semiaqueous titrimetry, in relation to the aqueous pH scale, is still controversial and under investigation (23).

The disadvantages, irregularities, and anomalies observed in the extrapolation technique, as well as the cautionary statements provided in various texts and reviews (20-22), indicate that there is a controversy surrounding the validity of the pKa values obtained by this method. There is no infallible and/or completely reliable method for relating the pH measurements obtained in semiaqueous solvents to the conventional aqueous pH scale. For this reason, when precise or thermodynamic values are of importance, spectrophotometric methods of determining acidity constants are the techniques of choice for sparingly soluble substances.

Ultraviolet Spectrophotometry

Ultraviolet or visible spectrophotometry is the ideal method of determining dissociation constants when the compound is too insoluble for potentiometry or when extremely weak acids or bases are under investigation (those acids or bases with a pKa value of greater than 11 or of less than 2). The spectral changes that are observed must be due entirely to the protonation or deprotonation at a site in conjugation with, or an inherent part of, a chromophoric group of the molecule (20,

21). The ratio of the molecular or neutral species to the ionized species is determined in a series of spectrally transparent buffers of either known or measured pH. The ratio of the two species is solely dependent upon the pH of the solution at the analytical wavelength.

The wavelength at which there is the greatest absorbance difference between the purely molecular and the purely ionized species is the analytical wavelength. If the compound obeys Beer's Law, the observed absorbance is the sum of the absorbances of all of the species present in the solution and a matched pair of quartz or silica cells (cuvettes) with a pathlength of 1 cm is most commonly used for this type of measurement.

The buffers that are used should be of a low and constant ionic strength (I=0.01) (20,40) and they should be nearly transparent in the ultraviolet region that is being examined. Solutions that are above an ionic strength of 0.1 M cannot be corrected for activity using the conventional Debye-Hückel Law (20) and deviations from Beer's Law may be exhibited above this particular ionic strength (21). Buffers can be adjusted to the appropriate ionic strength with sodium hydroxide or hydrochloric acid and they should not be stored in polythene containers as these may liberate an optically absorbing plasticizer (20). The reference solution must contain the same buffer components as the sample.

A convenient strength for a stock solution is 5×10^{-4} M, if solubility so permits, but stock solutions as weak as 2×10^{-5} M may be used for poorly soluble substances with a high absorptivity (20).

Acidic compounds may be dissolved in dilute base, and basic compounds in dilute acid, to facilitate their dissolution if their pKa values are below 10 and above 4, respectively (20). Alcohol can also be used in this regard, but its concentration should not exceed 1% in the final stock solution (20).

An ultraviolet spectrum of each solution is obtained over the desired wavelength range and the molecular and the ionized species are assumed to be isolated when there is no change greater than 1% in their absorbances with further pH changes. Medium effects on the spectra, decomposition of the solute, the presence of impurities, or the event of one or more ionization processes are indicated when the spectral curves fail to intersect at an isobestic point (20,21). If the compound is being chemically changed by either acidic or basic media, an alteration of the UV spectrum, with time, should be evident (20).

Once the molecular and the ionic species have been isolated, their absorbances are remeasured at the analytical wavelength. For an acid the absorbance of the anionic species is measured in a basic medium at 2 pH units above the pKa and the absorbance of the molecular species is measured in an acidic medium at 2 pH units below the pKa, while for a base an acidic medium provides the cation and an alkaline medium provides the molecular species.

The exact determination of the pKa is carried out using seven sample solutions which are appropriately diluted with buffers, the pH values of which are centered around the approximate pKa value with deviations of about ± 0.6 pH units (20). This total range of 1.5 pH units is necessary to detect any decomposition or any deviation from

Beer's Law that may occur (21) and thus one obtains a set of seven pKa values which are then averaged and should lie within ± 0.06 pKa units of the average pKa (20).

The treatment of the data involves the observed absorbances of the experimental solutions and the absorbances of the ionized and the molecular species, where the ratios of the deprotonated to the protonated species, in their logarithmic forms, are added to the measured pH values of the sample solutions. The final sums are equal to the negative logarithm of the acidity constant, the pKa, but the pKa values obtained in this manner are not thermodynamically rigorous even though the compound is present at great dilution. Activity corrections must be made because of the presence of buffer salts, the concentrations of which are used in these corrections. If the sample solutions are maintained at a constant ionic strength the corrections are easily performed and, providing that the ionic strength is between 0.01 and 0.1 M and the temperature is constant (20), the conventional Debye-Hückel Law is valid.

Certain disadvantages are also inherent in the ultraviolet spectrophotometric method. For example, the ionized species for very weak acids and bases is not always directly measurable but this problem can be solved by graphically estimating the absorbance of the ionized form (20,21). The major difficulty which is encountered with the use of the spectrophotometric method in determining acidity constants is the interference caused by a second ionization process when a compound with more than one ionizable group is being investigated. Overlapping dissociation constants, therefore, are those which are defined as being

less than 3 pKa units apart. The absorbance of the intermediate ionic species is not directly measurable and many modifications in the procedure and the calculations are required to develop a suitable protocol for determining overlapping acidity constants. Lewis (41) and Kappe and Armstrong (42) avoided the problem by determining the pKa values of phenolic amines using a combination of the potentiometric and spectrophotometric techniques.

General terminology which is suitable for dibasic acids, diacidic bases, and ampholytes describes diprotonated, monoprotonated, and nonprotonated species when more than one ionization process is involved. The absorbance of the monoprotonated species is not directly measurable, therefore, extensions of the spectrophotometric technique which circumvent this problem have been proposed. Bryson and Matthews (43) developed a relatively simple procedure for determining overlapping ionization constants which they illustrated with m-aminobenzoic acid as the model compound. At one particular analytical wavelength the nonprotonated and the diprotonated species must have similar absorbances which differ markedly from the absorbance of the monoprotonated species. One set of pH values representing the first ionization constant and another set representing the second ionization constant are, together with the absorbance values, substituted into two simultaneous equations that are graphically evaluated to obtain K_1 and K_2 values. The resulting intercepts yield values for the absorbance of the monoprotonated species but the procedure is only feasible when the absorbances of the nonprotonated and the diprotonated species are similar and this situation is, generally, not true with the majority of compounds.

Irving et al. (44) suggested a procedure that is applicable to systems in which the absorbances of the individual species of a substance are unknown or cannot be directly measured, and also to those situations where the acidity constants overlap. A series of successive approximations are involved and the 'best' values for the unknown parameters have been obtained when there is no significant change in these final values upon further calculation. The unknown parameters are usually the K_1 and K_2 values but the absorbances of the purely molecular or the purely ionized species can be calculated using a similar series of successive approximations.

Two additional modified spectrophôtometric methods for dibasic acids have been presented by Thamer (45). The procedures are applicable for any degree of overlap of the ionization constants and they do not require the direct absorbance measurements of the individual species encountered upon ionization. A minimum amount of data is required (only five points) and one of the methods directly calculates acidity constants without approximations while the other uses converging successive approximations.

Kokesh and Westheimer (46) treated the acidity constants as known values which were visually estimated from the plots of absorbance versus pH at the analytical wavelength of the substance under investigation. An iterative computer program was then used to calculate the best values of the molar absorptivities of the nonprotonated, monoprotonated, and diprotonated species. A slightly different pair of pKa values was then provided for repeated calculations, the end result being the refined values of these parameters which do not change upon

further calculation. Also, an appropriate titration curve can be fitted, by a least-squares analysis method, to the often scattered absorbance-pH data which are obtained when there is little difference between the molar absorptivities of the various species that are involved in the ionizations.

The representative class of methodology for determining overlapping ionization constants is characterized by the tedious calculations, suitable for computer programming, which are required. When the graph of absorbance versus pH of a compound indicates a region where the monoprotonated species predominates and the absorbances of the nonprotonated and diprotonated species are similar, the procedures of Thamer and Voight (47), Thamer (45), Ang (48), and Bryson and Matthews (43) are available. If the absorbance of the monoprotonated species and the overlapping acidity constants are the only variables or unknown parameters, a series of three simultaneous equations can be solved. This method has been extended by Thamer (45) and Roth and Bunnett (49) to include the absorbances of the nonprotonated and diprotonated species, which requires the solving of five simultaneous equations.

The methodology of Kokesh and Westheimer (46) displays an interesting version of an iterative computer program. Three simultaneous equations are solved for the absorbances of the nonprotonated, monoprotonated, and diprotonated species, whereas the acidity constants are treated as known values. It would seem obvious that if a parameter is directly measurable, its accuracy would be greater than that of the graphically estimated values of the ionization constants, although the

technique does have some merit when the total variation in the absorbance is small and the various chromophores are only slightly affected by the ionizations.

Albert and Serjeant (20) have also published a computer program that is dependent on the direct absorbance measurements of the nonprotonated and diprotonated species. Successive approximations are used to find the molar absorptivity of the monoprotonated species and the Ka_1 and Ka_2 values. This method uses the data uniformly, as well as separately, for the two ionization processes but the calculations are tedious and they incorporate the Debye-Hückel equation to apply activity corrections.

Heys et al. (50) have developed a least-squares computer method to obtain the values of overlapping acidity constants which uses all of the experimental data to solve for the molar absorptivity of the monoprotonated species and the Ka_1 and Ka_2 values. To find the 'best fit' of the absorbance-pH data a least-squares analysis, which yields the computed graph of absorbance versus pH, is used.

The ultraviolet spectrophotometric method, ideally suited for sparingly soluble compounds, does not always overcome solubility problems. Many medicinal substances are so insoluble in aqueous media that organic solvents must be added to aid dissolution. Albert and Serjeant (20) have suggested that an alcoholic content, which does not exceed 1% in the final stock solution, could be used for this purpose. This would translate into 0.2% ethanol in the sample solution that is ultimately to be examined. In order to aid the solubility of benzodiazepines, Barrett et al. (51) used traces of methanol in otherwise

aqueous solvent systems and their UV spectral information was later compared to the polarographic data obtained in methanol-water solvents by Clifford and Smyth (52). Smyth et al. (53) also examined the UV spectral data of SC-13504 (a pharmacologically active benzhydryl-piperazine derivative) obtained in nonaqueous solvents (methanol and cyclohexane) and also in 20% methanol. The methanol-water data was then used to calculate pKa_1 and pKa_2 values. Hurowitz and Liu (54) determined the pKa values of estrogens using aqueous solvent systems containing traces of ethanol, whereas Atwell et al. (55) examined dicationic analogs of the 4'-(9-acridinylamino)alkanesulfonamides in 20% dimethylformamide. Since only comparative or relative pKa values were desired for these antitumor agents, all of the ultraviolet spectrophotometric measurements were made using solvents of 20% dimethylformamide in aqueous buffers.

Fluorimetry

Since water miscible organic solvents were being employed to aid the dissolution of certain compounds, it is apparent that absorption spectrophotometry is not always the completely ideal technique for all sparingly soluble substances possessing functional groups which alter the UV spectra upon ionization. Fluorimetry is similar to UV spectrophotometry in that the relative intensity of fluorescence may reflect protonation or deprotonation when the sample solution pH is varied (21, 35,56,57). Relative pKa values are estimated graphically from a plot of the percent relative fluorescence versus pH and the pKa is equivalent to the pH at the point of 50% relative fluorescence.

Schulman et al. (35) obtained a fluorimetric pKa value for methaqualone which correlated well with the values obtained from UV spectrophotometric determinations (33,34) but the interpretation of fluorescence spectra in the determination of acidity constants is often difficult. The major criticism (21,56,57) is that, due to the altered electronic behavior, protonation and deprotonation in the excited molecule may differ from these same properties in the ground state. This should not constitute a problem since molecules irradiated with ultraviolet light become electronically excited when absorbing energy and the 'excited state' and ground state dissociations will occur, except in different pH ranges. Two inflection points will then be evident from the relative fluorescence versus pH profile and, consequently, a substance possessing native fluorescence in water is a suitable candidate for a fluorimetric pKa determination.

Structural changes intended to produce or enhance the fluorescence of a compound should be avoided since the acidity constant of the derivative, rather than the original compound, is then being examined. Poor aqueous solubility of the compound under investigation and/or the lack of native fluorescence in water are the factors that most frequently preclude the use of fluorimetry in the determination of acidity constants.

Nuclear Magnetic Resonance Spectrometry

Another spectrometric technique that has met with some success in the determination of acidity constants is nuclear magnetic resonance (NMR). The method has been employed when the compound under investigation

was too insoluble for potentiometry, its ultraviolet spectra did not exhibit any significant change upon ionization, or it was too weak as an acid or a base.

The technique involves the monitoring of the chemical shift, at various pH values, of nonexchanging protons that are affected by the ionization of a neighbouring functional group. This change in chemical shift is normally examined at room temperature but it has been found that at very low temperatures such as -89 and -120°C the proton-exchange rate slows down so that the actual site of protonation or deprotonation can be observed (21). To obtain NMR spectra at such low temperatures extraordinary solvents such as fluorosulfuric acid and antimony pentafluoride ('magic acid') are required but, although these super acids serve a useful purpose in identifying the site of ionization, they cannot be directly related to the aqueous pH scale and the determination of thermodynamic pKa values is not possible.

A plot of the chemical shift versus pH, of the nonexchanging proton near the ionizing group, gives a titration curve (20) and the pKa is calculated from the equation:

$$pKa = pH + \log \frac{\delta - \delta_o}{\delta_\infty - \delta},$$

where δ, δ_o, and δ_∞ are the chemical shifts of the representative group at the measured pH, in the deprotonated form, and in the protonated form, respectively (21). The pKa values of weak bases such as oxazole, and also of strong acids such as nitric acid, have been determined by the NMR spectrometric method (20).

The major disadvantage of the NMR spectrometric method of determining acidity constants is the high sample concentrations of approximately 0.1 M that are required for the measurements as this presents a problem for compounds that are sparingly soluble in water. The accuracy of the results is also limited because activity corrections are more difficult to apply, especially since an internal standard is used (21). Sophisticated instrumentation has enabled measurements at higher frequencies, the use of Fourier transform techniques, and computer accumulation to reduce the sample concentrations required for pKa determinations (21). The less concentrated solutions can then be maintained at a constant ionic strength by the addition of electrolytes such as NaCl and KCl (58).

Due to the expense and the lack of immediate availability of the aforementioned modifications, attempts to alleviate the solubility problems have involved, chiefly, water miscible organic solvents to aid in solubilizing the sample (21). This appears to be the typical solution and it has been used, for the same purpose, in both the potentiometric and the UV spectrophotometric methods of determining acidity constants. The validity of the results, in relation to the aqueous pH scale, can again be questioned.

NMR spectrometry has been found to be particularly useful, when solubility so permits, for the determination of overlapping ionization constants and the calculation of microscopic dissociation constants. If more than one ionizable group is present, the shift of one group is expected to begin before the shift of the other group begins but this becomes more complex when the ionizations strongly overlap because then simultaneous shifting of both of the groups will occur

(59). Also, if there is no overlap of the ionizations, the microscopic dissociation constants are identical to the macroscopic acidity constants but if the two ionizations overlap, the macroscopic constants are composed of the microscopic constants (58). Generally, if the ionization of two or more groups occurs simultaneously, the macroscopic constants are composed of the microscopic constants for the ionization of the individual groups (59).

When examining compounds with more than one ionizable group, the chemical shifts of carbon-bonded protons at sites near the ionizing groups are observed (59) and the chemical shift of an adjacent group may depend on the degree of protonation of only one of the ionizing groups (a unique resonance) or on both of the ionizing groups (a common resonance) (58).

Formerly, microscopic dissociation constants were difficult to determine and, instead, macroscopic acidity constants were employed in calculating these values. For example, the microscopic dissociation constants of the amino acid cysteine were calculated using the macroscopic acidity constants of cysteine and some of its derivatives in which the sulfhydryl and amino protons were replaced by alkyl groups. As discussed by Rabenstein (59), this method of calculating microscopic constants is of limited accuracy because the assumption that replacing the protons with alkyl substituents did not affect the acidity of the other protons is not necessarily valid.

Kesselring and Benet (60) determined the macroscopic and microscopic dissociation constants of isochlortetracycline by both potentiometry and NMR spectrometry. Also, the three acidic dissociation

constants of the tetracyclines, the last two of which strongly overlap, were assigned to particular functional groups. Experimental conditions included a 50-50 w/w methanol-water solvent system, a probe temperature of 25 ± 0.5°C, and drug concentrations of 0.007-0.017 M. The same sample solutions were used to determine the stoichiometric dissociation constants, as well as to measure chemical shifts on a high resolution NMR spectrometer. The percentage of ionization for a site was then calculated by dividing the chemical shift of the chosen neighbouring protons by the total shift of this group over the entire titration.

Rabenstein and Sayer (58) utilized a unique resonance to examine the fractional deprotonation, as a function of pH, of the two ionizable ammonium groups of lysine and of ethylenediaminemonoacetic acid. The microscopic dissociation constants from this fractional deprotonation data were evaluated by a nonlinear least-squares curve fitting method which makes use of macroscopic acidity constants determined from the same NMR measurements.

Nuclei other than hydrogen, such as fluorine-19, phosphorous-31, and carbon-13, have been used, in relation to nearby protonations, for pKa determinations (21). Sayer and Rabenstein (61) employed both ^{13}C and ^{1}H nuclear magnetic resonance to determine the macroscopic acidity constants for 2,3-diaminopropionic acid (dap), 2,4-diaminobutyric acid (dab), ornithine (orn), and lysine (lys). The ^{13}C spectra were scanned at an operating frequency of 22.63 MHz using the Fourier transform technique and the chemical shift titration curves for alkylcarbon atoms were obtained with the ^{13}C nuclei of dioxane as the internal standard.

The applicability of the NMR spectrometric method of determining pKa values is reduced by the high sample concentrations required for the measurements. The sophisticated equipment that is necessary for the scanning of less concentrated sample solutions is frequently impractical and/or not readily available. Resorting to the use of water miscible organic solvents, in an attempt to solubilize sparingly soluble compounds, yields relative or comparative pKa values only as the ordinarily appropriate activity corrections may not be applicable when semiaqueous solvent systems are involved. Although thermodynamic pKa values are frequently difficult to obtain, the NMR spectrometric technique is a valuable tool for identifying the site of ionization and, if solubility so permits, the calculation of microscopic dissociation constants.

Solubility Variation with pH

Solubility variation with pH is a suitable method of determining dissociation constants for substances that are too insoluble for aqueous potentiometry or nuclear magnetic resonance spectrometry, or for those compounds which lack appropriate ultraviolet spectra or do not possess native fluorescence in water. The observed solubility, at any given pH, is related to the solubility of the neutral molecule and the solubility of the salt. The solubility of the neutral molecule is the intrinsic solubility and this parameter is measured in hydrochloric acid for an acid and in sodium hydroxide for a base.

The procedure of Krebs and Speakman (62), together with details compiled by Albert and Serjeant (20), commences with the measurement

of the intrinsic solubility of the compound at three different pH values. For acids, 0.01 N HCl is utilized and 0.01 N NaOH is the choice for bases. The value is then checked by measuring the intrinsic solubility at one half of a pH unit both above and below that of the original solution and these three values of the intrinsic solubility should agree to well within the range of the experimental error involved in the measurements. The solubility of the compound under investigation is then determined at a pH value near that of the suspected pKa and an approximate pKa value is calculated. The solubilities of a set of seven sample solutions, the pH values of which are centered around the approximate pKa within a range of ± 1 pKa unit, are determined and the final pKa values are calculated.

The data are treated in a manner similar to those obtained by the potentiometric and spectrophotometric techniques. The relationships between the observed solubility (S_o'), the intrinsic solubility (S_i), the pH, and the pKa are expressed by the following equations:

$$pKa = pH - \log\left[\frac{S_o'}{S_i} - 1\right] \qquad \text{for acids, and}$$

$$pKa = pH + \log\left[\frac{S_o'}{S_i} - 1\right] \qquad \text{for bases.}$$

Corrections for hydrogen or hydroxyl ion activity are not necessary since stoichiometric concentrations are not used (20), however, it is suggested that the ionic strength should be kept constant and this can be achieved by the use of a swamping electrolyte such as sodium chloride or potassium chloride (20,21). Since a substance may be very soluble at

certain pH values, the resulting ions may contribute to the ionic strength of the solution and even with the use of a swamping electrolyte thermodynamic pKa values are not believed to be obtainable by the solubility variation with pH method (20,21).

For ampholytes or compounds that do not give consistent intrinsic solubilities, a graphical method is available where the observed solubility is plotted against the reciprocal of the hydrogen ion activity and the straight line is extrapolated to $1/\{H^+\}=0$. The point of intersection with the abscissa yields the intrinsic solubility.

The practical aspects of solubility studies are of interest since the techniques that have been used are numerous and varied. Generally, an excess of the unknown compound is agitated in a thermostatic bath with the appropriate buffer solution under an inert atmosphere, usually nitrogen, until there is no longer any change in the concentration, with time. The undissolved solid is then separated from the suspension and the pH of the clear solution is measured. The system can be maintained at any temperature as long as it is held at a constant value because an increase in temperature usually enhances solubility, therefore, this parameter must be carefully controlled.

The greatest variations in methodology pertain to the time period of agitation and equilibration, as well as the method of separating the excess solid from the solution. Gilligan and Plummer (63) and Biamonte and Schneller (64) analyzed sulfonamides by shaking suspensions for 18 hours and then filtering them by gravity through Whatman No. 1 paper. Lordi and Christian (65) agitated a number of antihistamines for 30 minutes, allowed them to equilibrate for 15 minutes, and then

added buffer until the cloudy solutions cleared. The solutions were then further equilibrated for an hour, with occasional shaking and, finally, they were filtered through wetted, coarse sintered glass funnels.

Forist and Chulski (66) agitated suspensions of tolbutamide and its metabolite for 18-20 hours and filtered the saturated solutions by means of a positive-pressure-constant-temperature-filtering device, whereas Baer et al. (12) made supersaturated solutions of chlorothiazide at elevated temperatures, allowed them to equilibrate at room temperature, and then filtered the solutions. Schill (67,68) analyzed several amines and quaternary ammonium compounds by shaking suspensions in a closed system, or under a nitrogen atmosphere, at a constant temperature for 24 hours and then centrifuging the solutions. In addition, Schill (67,68) used the exceptional technique of allowing a solvent to slowly percolate over a column of solid drug and this process permitted a slow saturation of the solution.

In an attempt to obtain solubility data Nygard et al. (69) agitated suspensions of salicylazosulfapyridine for 30 minutes and then centrifuged the samples, whereas Green (70), in his work on pKa determinations, studied the solubilities of various aminoalkylphenothiazine tranquillizers and related compounds by a simple technique. Suspensions were shaken for 3 hours and the undissolved material was removed by centrifugation. Furthermore, Green (70) employed the turbidity method, which is a variation of the solubility method and involves the shaking of suspensions containing known concentrations of drug. The absorbances of the suspensions are then measured at a wavelength

at which the compound normally does not absorb significantly. The observed absorbance is due to the scattering of light by the turbid suspension and a plot of this absorbance versus concentration gives two straight lines that intersect at the point of solubility.

Solubility studies of estradiol by Ruchelman and Haines (71) were approached from the standpoint of supersaturation in that suspensions of the drug were periodically agitated at an elevated temperature for 3 days and then held for 3 days at the desired temperature. Samples were withdrawn by using pipettes with wool plugs in the constriction, which filtered out any crystalline material. Hou and Poole (72) allowed suspensions of penicillins to mechanically rotate for 2 hours, then to equilibrate for 30 minutes, and samples of the supernatants were filtered through Millipore filters, whereas Hähnel (73) examined the solubility properties of 17β-estradiol by agitating suspensions at regular intervals for 20-24 hours and then filtering the samples through sintered glass. The difference was found to be insignificant when centrifugation, rather than filtration, was the method of separation of any excess solid from the suspensions.

Albert and Serjeant (20) found that 3 hours of continuous agitation was sufficient for sulfadiazine when determining its pKa value. Le Petit (74), in the determination of the pKa value of medazepam, achieved equilibration by intermittently shaking the suspensions and filtering the supernatant. To accomplish the same purpose Hurwitz and Liu (54) vigorously stirred suspensions of numerous estrogens for 4-24 hours and then filtered aliquots through 0.2 μm Nucleopore filter membranes.

In summary of the preceding discussion on solubility studies, it is apparent that the agitation and equilibration steps have been carried out in very diverse manners. The ideal methodology would appear to involve the continuous agitation of suspensions, at a constant temperature, in closed vessels or under a nitrogen atmosphere for periods of time varying from 15 minutes to 3 weeks, depending upon the compound under investigation. The suspensions should then be equilibrated for approximately one hour, following which samples are filtered and analyzed for drug content. This procedure is repeated until there is no further change in the solubility. The method that has been described is rather tedious and requires long periods of time to complete. Despite this disadvantage, however, the solubility method has experienced an increase in popularity because of its suitability for certain medicinal compounds since many drugs are characterized by poor aqueous solubility.

Determinations of the solubility properties of the sulfonamides were frequently performed because sulfonamide chemotherapy often resulted in kidney damage due to the low solubility of these agents, and their N^4-acetyl metabolites, in acidic urine. This finding prompted further solubility studies of drugs with one or more sulfonamido group, as well as drugs that exhibited poor solubility in aqueous media at a similar pH to that of urine (63,64,66). Such investigations can be carried out, at the appropriate temperature, in buffers of physiological pH and the solubility data that are obtained can also be used to determine the pKa values of the compounds being examined.

Various convenient techniques, such as colorimetry (64), ultraviolet spectrophotometry (54,66,70,74), and gas-liquid chromatography

(54,71), can be employed in the analysis of the samples obtained from solubility studies. High pressure liquid chromatography is also useful as an analytical method. For all of the aforementioned techniques, the standard curves do not need to be derived from aqueous solutions of the drug as only the concentration, which is not influenced by the solvent, is being analyzed. Once the sample solution pH has been measured, the aliquot prepared for the analysis can be appropriately diluted with a nonaqueous solvent and the drug content can be measured by a suitable technique.

The difficulty in obtaining thermodynamic values has been the major reason for not employing the solubility variation with pH method for pKa determinations. Comparisons of solubility-pH and ultraviolet spectrophotometric pKa determinations have indicated that good agreement, for the pKa values of several drugs, exists between the two methods (66,74). Green (70) found his values to be in good agreement with the extrapolation values obtained by Chatten and Harris (31) for selected phenothiazines. Peck and Benet (75) described a method for determining thermodynamic pKa values but their agitation and equilibration procedure is similar to the laborious techniques described previously. Agitation for long periods of time is followed by the filtration of sample aliquots through 0.22 μm filter membranes and the appropriately diluted sample is then analyzed by UV spectrophotometry. The procedure for a given sample is completed when a solubility change of less than 3% is observed in three successive 24-hour checks. The apparent pKa values are corrected for activity by the calculation of the ionic strength of each solution, which ultimately yields

thermodynamic values. A computer provides the optimum means of performing the calculations.

Partition or Distribution Studies

A variation of the solubility technique is provided by the partition method, which involves the partitioning of a compound between immiscible aqueous and organic phases. The sparingly soluble compound is dissolved in a suitable organic solvent that is immiscible with water and as the pH of the aqueous phase changes the solubility characteristics of the solute also change. Basic compounds readily partition into the aqueous phase at low pH values, whereas the solubility of acidic compounds increases in basic aqueous phases. The calculations that are performed are similar to those for solubility data since the molecular and the ionized species are being compared, in relation to the solution pH.

Suitable methods of analysis are available for the solubilized species and these include ultraviolet spectrophotometry, gas-liquid chromatography, high pressure liquid chromatography, and colorimetry. Just as in the solubility method, the sample can be derivitized for analysis because only the concentration, which is independent of solvent effects, is being examined. Both the aqueous and the organic phase can be analyzed for drug content by gas-liquid chromatography and high pressure liquid chromatography, and this same principle applies to UV spectrophotometry if an organic solvent which is transparent to UV light is used. A known quantity of the compound under investigation can be partitioned between immiscible solvents and both of the phases can be

subsequently analyzed for drug content and this procedure could provide an assurance of the accuracy of the method.

The choice of the solvent for the organic phase is of considerable importance but the selection is complicated by the significant solubility or miscibility of water with many organic solvents (21). Although essentially immiscible with water, a fractional proportion of the selected organic solvent may be miscible with the aqueous phase and, clearly, the assumption that only the neutral or molecular form of a compound partitions into the organic solvent becomes invalid if the miscibility between the aqueous and organic phases is significant. The possibility of the compound under study acting as a cosolvent would contribute to the error already generated by the apparent 'miscibility' and, furthermore, thermodynamic pKa values cannot be obtained if the accuracy of the method is questionable. The assumption that the aqueous buffers and buffer salts do not fractionally partition into the organic phase becomes invalid when activity corrections are to be applied.

Dissociation Constants of Benzothiadiazines

As has been demonstrated, the selection of an appropriate method for determining acidity constants involves a critical examination of both the physico-chemical properties of the compounds and the numerous techniques available to determine dissociation constants. When sparingly soluble drugs, such as the benzothiadiazines, are under investigation the number of available choices is limited to those methods which are amenable to compounds that possess limited solubility in water. Regarding the benzothiadiazines specifically, methods that have been

utilized to determine their dissociation constants include aqueous and semiaqueous titrations, ultraviolet spectrophotometry, and solubility variation with pH. The first acidic dissociation constant (referred to as pKa_1) has been of considerable interest while the second acidic dissociation constant (referred to as pKa_2) was presumed not to be of physiological importance (18,19).

Benzothiadiazines are amphoteric compounds which possess one basic and two acidic ionizable functional groups but behave essentially as dibasic acids. The overlap between pKa_1 and pKa_2 presents only one obstacle in the determination of these ionization constants. The overlap does not allow for a simple conductimetric, potentiometric, or spectrophotometric technique and the problem is compounded by the extremely low aqueous solubility of the benzothiadiazines.

Values obtained by aqueous potentiometric titration were reported by Mollica et al. (13), Essig (19), and Whitehead et al. (7). Mollica et al. (13) employed the method of Noyes (20) for the overlapping pKa values of hydrochlorothiazide but carried out the titration at 60°C. It is assumed that the experimental values quoted by Whitehead et al. (7) and Charnicki et al. (11) for chlorothiazide, and by the Merck Index (76,77) for hydrochlorothiazide and hydroflumethiazide, are valid at room temperature.

Essig (19) was only concerned with the pKa_1 and titrated 0.001 M concentrations of chlorothiazide, flumethiazide, hydrochlorothiazide, and hydroflumethiazide with a basic titrant. This procedure, according to Albert and Serjeant (20), requires a refinement in both the apparatus and the calculations but results are not satisfactorily reproducible

below a concentration of 0.005 M. The method may provide reasonably accurate pKa values for the few soluble benzothiadiazines but it is not applicable to the majority of these agents.

For the very sparingly soluble derivatives, their dissolution in water miscible organic solvents, followed by potentiometric titration, has been considered. Semiaqueous potentiometric titrations in 66% dimethylformamide, carried out by Whitehead et al. (7,8) for various benzothiadiazines, yielded relative acidity constants. Novello and Sprague (78) obtained values of $pKa_1 = 9.1$ and $pKa_2 = 10.5$ for cyclothiazide by measuring the pH at the point of half neutralization in 30% methanol. An extension of the semiaqueous titrimetric method, which involves the extrapolation of acetone-water mixtures to 100% water, provided a pKa value of 9.4 for the acidic dissociation constant of methyclothiazide (79). Moskalyk et al. (80) have experimented further with this procedure to obtain the pKa values of other benzothiadiazines.

Mollica et al. (13) attempted to use the ultraviolet spectrophotometric method but they could only obtain the pKa_1 values of hydrochlorothiazide and two of its analogs by this technique. The resultant values do provide some insight into the effect of substitution on the acidic ionization constants of the benzothiadiazines.

Ågren and Bäck (18) investigated solubility variation with pH and obtained a pKa_1 value of 8.53 ± 0.05 for bendroflumethiazide. They cited their technique as being the method of Green (70) and, therefore, did not present any details of their procedure.

This constitutes a concise overview of the available literature information regarding the possible methods of determining the acidity constants of sparingly soluble compounds, as well as the reported pKa values of the benzothiadiazines and the methods used in obtaining these constants. A summary detailing the literature values of the dissociation constants of the benzothiadiazines most commonly used in medical practice is reported in Table 1.

Table 1. Literature Values of Benzothiadiazine Dissociation Constants

Benzothiadiazine	Method of Determination									
	Aqueous Potentiometric Titration			Semiaqueous Potentiometric Titration			Ultraviolet Spectrophotometry			Solubility Variation with pH	
	pKa_1	pKa_2		pKa_1	pKa_2		pKa_1	pKa_2		pKa_1	pKa_2
chlorothiazide	6.7	9.5	(11)	6.9	12.1	(7)					
	6.8	9.4	(7)								
	6.83	-	(19)								
flumethiazide	6.44	-	(19)	6.3	-	(80)					
hydrochlorothiazide	7.0	9.2	(77)	8.7	-	(80)	8.7	-	(13)		
	7.9	9.2	(76)								
	8.6	9.9	(13)								
	8.80	-	(19)								
hydroflumethiazide	8.9	10.7	(77)	8.5	-	(80)					
	8.45	-	(19)								
cyclothiazide				9.1	10.5	(78)					
				8.8	-	(80)					
trichloromethiazide				6.9	-	(80)					
bendroflumethiazide										8.53	- (18)
methyclothiazide				9.4	-	(79)					
				9.5	-	(80)					
polythiazide				9.05	-	(80)					

STATEMENT OF THE PROBLEM

A vast majority of the drugs commonly used in medical practice can be labeled as 'sparingly soluble' (22, 30, 37) and this physical property has prevented the rapid, as well as the accurate, determination of their ionization constants. Since the acidity constants of drugs are often of great importance biologically (21, 22, 51), the availability of a suitable method for determining these values is a necessity. The resultant search for an adequate method of determining thermodynamic dissociation constants for sparingly soluble substances appears to have produced an ideal version of the solubility variation with pH technique. Recently Peck and Benet (75) published a procedure that encompasses all of the possibilities encountered upon ionization. These include polyprotic and amphoteric compounds, plus overlapping ionization constants.

The dissociation constants of the benzothiadiazines have been particularly difficult to determine due to numerous complicating factors such as low aqueous solubility, an amphoteric nature, overlapping acidity constants, and instability in alkaline media. Decomposition occurs in media of pH 8 or greater and is enhanced as the pH and/or the temperature increases (5, 11-13).

The purpose of this work is to determine, wherever possible, the thermodynamic pKa_1 and pKa_2 values of a series of benzothiadiazine diuretics. A literature survey has revealed numerous dissimilar pKa values which were determined by very diverse methods. An effort has been made to analyze all of the chosen benzothiadiazines by the same method, ultraviolet spectrophotometry. The validity of the

literature dissociation constants has been examined and, also, comparisons with the ultraviolet spectrophotometrically determined values are presented. The investigation of the dissociation constants has been completed with an examination of the effects of substitution on the ionization constants of the benzothiadiazines.

All but two of the selected compounds have overlapping acidic pKa values. The acidic protons are in different chemical environments and, therefore, an attempt to determine the order in which they dissociate has also been made. From the literature, only comparisons to N-formylsulfonamides (7,9,19) have been offered in postulating the order of deprotonation. This work provides further comparisons involving two benzothiadiazines, which possess only one acidic dissociation constant each, and diazoxide. Diazoxide is structurally similar to the benzothiadiazine diuretics but shares only their antihypertensive activity (1,2). Furthermore, ultraviolet spectrophotometry and nuclear magnetic resonance spectrometry have been included in an effort to clarify the order of deprotonation of the acidic hydrogens.

Finally, suggestions for the improvement and refinement of the presented technique have been offered. Preliminary methodology for further research in determining the dissociation constants of the benzothiadiazines has also been proposed. Both of the proposed techniques, ultraviolet spectrophotometry and solubility-distribution studies, can subsequently be applied to other sparingly soluble drugs.

EXPERIMENTAL

Apparatus

Standard laboratory glassware

Mettler Gram-atic analytical balance

Thomas Hoover Melting Point Apparatus

Perkin-Elmer 267 Grating Infrared Spectrophotometer

Hewlett-Packard 5981A Mass Spectrometer

Eastman Chromagram Developing Apparatus and Sheets (silica gel adsorbent with fluorescent indicator)

Ultraviolet lamp (Applied Science Laboratories, Inc.)

Dubnoff Metabolic Shaking Incubator (GCA/Precision Scientific)

Servall refrigerated-automatic centrifuge

Hellma quartz cuvettes (1 cm)

Unicam SP 1800 ultraviolet spectrophotometer and recorder

Beckman Model 25 spectrophotometer and recorder

Fisher Accumet Model 320 expanded scale pH meter (fitted with glass and calomel electrodes)

PDP 11-05 Minicomputer (Digital Equipment Corporation, Maynard, Mass.)

Bruker WH-200 Nuclear Resonance Spectrometer

Reagents

All chemicals and reagents employed in the experimental procedures were either A.C.S. or reagent grade in quality, unless otherwise specified.

Buffers

Buffer solutions were prepared with glass distilled and deionized water, free of carbon dioxide. Table 2 provides further information pertaining to the pH range, the composition of the stock solutions, and the ionic strength of the buffers used in this investigation.

Table 2. Buffers Transparent to Ultraviolet Light

Buffer	pH Range	Composition of Stock Solutions* Solution A	Solution B	Ionic Strength
Phosphate (Sorensen)	5.0 - 8.0	KH_2PO_4 1/15 M	$Na_2HPO_4 \cdot 2H_2O$ 1/15 M	variable
Sodium borate/HCl (Sorensen)	7.8 - 9.2	$Na_2B_4O_7$ 0.05 M	HCl 0.1 N	0.1
Sodium borate/NaOH (Sorensen)	9.4 -10.6	$Na_2B_4O_7$ 0.05 M	NaOH 0.1 N	0.1
Glycine/NaOH (Sorensen)	8.6 -12.8	glycine 0.1 M in NaCl 0.1 N	NaOH 0.1 N	0.1
Succinate	3.60- 6.20	succinic acid 0.1 M (pH 3.60-4.70)	KOH 0.05 M	0.01
		succinic acid 0.02 M (pH 4.80-6.20)	KOH 0.05 M	0.01
Phosphate	6.40- 7.70	KH_2PO_4 0.02 M	Na_2HPO_4 0.01 M	0.01
Trihydroxymethyl-aminomethane (TRIS)	7.70- 8.90	TRIS 0.1 M	HCl 0.1 M	0.01
Borate	8.50- 9.70	$Na_2B_4O_7$ 0.025 M	H_3BO_3 0.1 M (pH 8.50-9.20)	0.01
		$Na_2B_4O_7$ 0.025 M	KOH 0.01 M (pH 9.30-9.70)	0.01
Carbonate	9.60-10.60	$NaHCO_3$ 0.02 M	Na_2CO_3 0.01 M	0.01

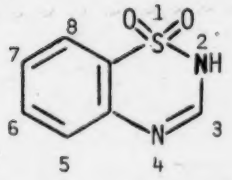
* The amounts of the stock solutions required to make up a buffer solution of the desired pH value are given in references (40) and (81).

Reference Standards

Purification of the selected benzothiadiazines listed below was achieved by recrystallization to a constant melting point.

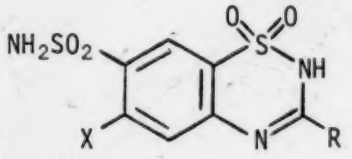
Drug	Manufacturer
chlorothiazide	Merck Sharp and Dohme Canada Limited
flumethiazide	E.R. Squibb and Sons of Canada Limited
benzthiazide	A.H. Robins Company Limited
hydrochlorothiazide	Merck Sharp and Dohme Canada Limited
hydroflumethiazide	Bristol Laboratories of Canada
bendroflumethiazide	E.R. Squibb and Sons of Canada Limited
cyclothiazide	Eli Lilly and Company
cyclopenthiazide	CIBA Pharmaceutical Company
althiazide	Pfizer Company Limited
trichloromethiazide	Schering Corporation Limited
methyclothiazide	Abbott Laboratories Limited
polythiazide	Pfizer Company Limited
diazoxide	Schering Corporation Limited

Summary of Data for Benzothiadiazines and Diazoxide



2H-1,2,4-benzothiadiazine-1,1-dioxide ring system

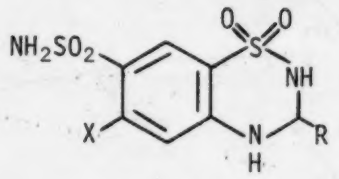
1. Thiazides



Generic Name	R	X	MW	mp*(°C)	Recrystallization Solvent
chlorothiazide	-H	Cl	295.74	>300	ethanol
flumethiazide	-H	CF_3	329.28	>300	ethanol-water
benzthiazide	$-CH_2S$-benzyl	Cl	431.96	241-5	ethanol-acetone-water

* All compounds melted with some degree of decomposition and three recrystallizations of the reference standards were performed when melting points were greater than 300°C.

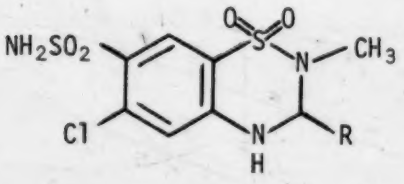
2. Hydrothiazides



Generic Name	R	X	MW	mp*(°C)	Recrystallization Solvent
hydrochlorothiazide	-H	Cl	297.72	267-270	ethanol-water
hydroflumethiazide	-H	CF_3	331.92	271-273	ethanol-water
bendroflumethiazide	-benzyl	CF_3	421.41	222-224	ethanol
cyclothiazide	-norbornylenyl	Cl	389.91	231-232	ethanol-water
cyclopenthiazide	$-CH_2$-cyclopentyl	Cl	379.89	236-240	ethanol-water
althiazide	$-CH_2SCH_2CH{=}CH_2$	Cl	383.91	196-199	ethanol-water
trichloromethiazide	$-CHCl_2$	Cl	380.67	271-272	ethanol-acetone-water

*All compounds melted with some degree of decomposition.

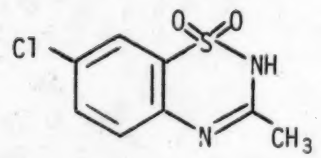
3. N^2-Methylhydrothiazides



Generic Name	R	MW	mp*(°C)	Recrystallization Solvent
methyclothiazide	$-CH_2Cl$	360.25	218-219	ethanol
polythiazide	$-CH_2SCH_2CF_3$	439.90	211-215	ethanol-water

*All compounds melted with some degree of decomposition.

4. Diazoxide



MW 230.70

mp > 300°C

recrystallized three times from ethanol

Procedures

A. Preparation of Reference Decomposition Products

The benzenedisulfonamide decomposition products of bendroflumethiazide and cyclothiazide were prepared by dissolving approximately 1 g of drug in 30 ml of 20% NaOH solution. The mixture was refluxed for 2 hours, allowed to stand for 20 hours, and then acidified to pH 4 with 6 N HCl. The resulting precipitate was separated by vacuum filtration, washed with distilled water, and recrystallized from water to a constant melting point.

1. 4-Amino-6-trifluoromethyl-1,3-benzenedisulfonamide (I)

Prepared from bendroflumethiazide; fine white crystals (yield 0.52 g or 68.0%) melted at 245-247°C with decomposition [literature (5) mp 246-247°C]. R_f value (ethylacetate:benzene): 0.30 and reacted with acidified p-DMAB to produce lemon yellow spots.

Infrared Spectrum (KBr disc)

3460 cm^{-1}	(N-H stretch of primary aromatic amine)
3370 and 3280 cm^{-1}	(N-H stretch of sulfonamido group)
1625 cm^{-1}	(N-H bend of primary aromatic amine)
1315 and 1145 cm^{-1}	(S=O stretch of sulfonamido group)

Mass Spectrum: m/e (% relative abundance)

(M^+)	319 (19.5)	175 (19.0)
	303 (4.7)	174 (18.4)
	302 (6.8)	159 (10.0)
	255 (7.9)	158 (22.1)
	239 (5.3)	80 (94.7)
	176 (17.9)	

2. 4-Amino-6-chloro-1,3-benzenedisulfonamide (II)

Prepared from cyclothiazide; coarse yellow crystals (yield 0.45 g or 61.3%) melted at 254-255°C with decomposition [literature mp (4) 251-252°C and (5) 259-260°C]. R_f value (ethylacetate : benzene): 0.28 and reacted with acidified p-DMAB to produce lemon yellow spots.

Infrared Spectrum (KBr disc)

3460 cm^{-1}	(N-H stretch of primary aromatic amine)
3370 and 3290 cm^{-1}	(N-H stretch of sulfonamido group)
1635 cm^{-1}	(N-H bend of primary aromatic amine)
1320 and 1160 cm^{-1}	(S=O stretch of sulfonamido group)

Mass Spectrum: m/e (% relative abundance)

(M^+ Cl^{37})	287 (40.9)	268 (90.9)	125 (40.9)
(M^+ Cl^{35})	285 (100.0)	223 (15.9)	124 (56.8)
	271 (13.6)	221 (40.9)	114 (43.0)
	270 (40.9)	142 (50.0)	105 (50.0)
	269 (31.8)	129 (45.5)	

3. 4-Amino-2-chloro-5-(methylsulfamyl)-benzenesulfonamide (III)

Prepared from polythiazide by Moskalyk et al.(82). Their procedure differed only in the extraction of the acidified product into ethylacetate and the removal of the organic solvent using a minimum of heat. The fine off-white crystals melted at 170°C with decomposition [literature mp (5) 168-170°C]. R_f value (ethylacetate: benzene): 0.46 and reacted with acidified p-DMAB to produce lemon yellow spots.

B. Detection of Benzothiadiazine Decomposition by Thin Layer Chromatography

To ensure the purity of the prepared benzenedisulfonamides the recrystallized products were dissolved in ethylacetate (approximately 1 mg/ml) and 30 µl aliquots were applied to microscope slides (7.5 cm x 2.5 cm) coated with a 250 micron layer of silica gel G adsorbent containing a fluorescent indicator. The spots did not exceed 5 mm in diameter and the microscope slide TLC plates were activated at 100°C for 1 hour before use. The TLC plates were developed for 5-10 minutes in an ethylacetate:benzene (8:2) solvent system, air dried, examined under short wave (254 nm) UV light, and finally sprayed with acidified p-DMAB reagent. This spray reagent was prepared as follows: 1 g of p-dimethylaminobenzaldehyde, dissolved in 95% ethanol to provide a 1% solution, was acidified by the addition of 10 ml of concentrated HCl.

The 12 selected benzothiadiazines, the reference decomposition products, and diazoxide were analyzed by thin layer chromatography. Each compound was dissolved in ethylacetate (approximately 1 mg/ml), but for those compounds not completely soluble, the undissolved portion was allowed to settle and the supernatant was used in all of the chromatographic investigations. This procedure was considered to be acceptable for qualitative purposes.

Eastman Chromagram Sheets were spotted with 10 µl aliquots of the ethylacetate stock solutions, the spots not exceeding 5 mm in diameter. These chromatographic sheets (20 cm x 20 cm) are precoated with silica gel adsorbent containing a fluorescent indicator and the adsorbent coating of 100 micron thickness is on an inert, flexible poly-(ethyleneterephthalate) support of 200 micron thickness. Poly-acrylic acid is the added binder and the fluorescent indicator is lead-manganese-activated calcium silicate.

The chromatographic sheets were developed in an Eastman Chromagram Developing Apparatus containing an ethylacetate : benzene (8:2) solvent system for 1 1/2 hours. The sheets were then air dried and examined under short wave (254 nm) UV light, the visualized spots were marked, and their R_f values were calculated using the conventional formula:

$$R_f = \frac{\text{distance travelled by solute}}{\text{distance travelled by solvent}} .$$

Finally, the TLC sheets were sprayed with acidified p-DMAB reagent.

Summary of Data for Thin Layer Chromatographic Analysis

Compound	R_f Value in Ethylacetate : Benzene (8:2)	Color Reaction with Acidified p-DMAB
hydrochlorothiazide	0.17	-
hydroflumethiazide	0.20	-
cyclothiazide	0.56	-
cyclopenthiazide	0.57	-
trichloromethiazide	0.27	-
althiazide	0.55	-
bendroflumethiazide	0.68	-
chlorothiazide	0.05	-
flumethiazide	0.18	-
benzthiazide	0.08	-
diazoxide	0.11	-
polythiazide	0.60	-
methyclothiazide	0.42	-
benzenedisulfonamide (I)	0.30	lemon yellow
benzenedisulfonamide (II)	0.28	lemon yellow
benzenesulfonamide (III)	0.46	lemon yellow

C. pKa Determination by Solubility Variation with pH

The method of Green (70), as interpreted from the reports of Ågren and Bäck (18), was used to determine the pKa values of bendroflumethiazide and methyclothiazide.

1. Standard Curves

A 1×10^{-4} M stock solution of methyclothiazide was prepared in 95% ethanol and diluted with buffers of the appropriate pH values to yield

the standard concentrations, as described by the following scheme:

Concentration (moles/l)	95% Ethanol (ml)	Methyclothiazide 1×10^{-4} M Stock Solution (ml)
1×10^{-5}	0.00	2.50
8×10^{-6}	0.50	2.00
6×10^{-6}	1.00	1.50
2×10^{-6}	2.00	0.50
1×10^{-6}	2.25	0.25
8×10^{-7}	2.30	0.20
6×10^{-7}	2.35	0.15
4×10^{-7}	2.40	0.10
2×10^{-7}	2.45	0.05

The methyclothiazide stock solution and the 95% ethanol were added with a 5 ml burette graduated in 0.01 ml units and equipped with a platinum tip. Sufficient buffer was added to make the volume equal to 25 ml, thus the final standard concentrations contained approximately 10% ethanol. Individual buffers in the pH range of 8.6-11.0 were prepared at 0.4 pH unit intervals from glycine/NaOH (Sorensen) stock solutions and the ionic strength was maintained at 0.1 M in all instances.

The absorbances at the wavelength of maximum absorption for each pH, as well as at the isobestic point, were obtained using a Beckman Model 25 spectrophotometer. The isobestic point for methyclothiazide occurs at 267 nm, as determined from Figure 34.

2. Solubility Determinations

To determine their pKa values, the solubility variation with pH change of bendroflumethiazide and methyclothiazide was studied.

a. Methyclothiazide

An excess of the pure drug was shaken with 25 ml of the glycine/NaOH (Sorensen) buffer of the appropriate pH and agitated for 15 minutes in a Dubnoff Metabolic Shaking Incubator maintained at a constant temperature of 25 ± 1°C. After 5 minutes of equilibration the suspensions were filtered through Whatman #1 filter paper and the clear filtrate was centrifuged at 15,000 g for 10 minutes at 25 ± 1°C. Immediately following centrifugation the pH of the solutions was obtained with a Fisher Accumet Model 320 expanded scale pH meter and the absorbances were read at 267 nm with the buffer serving as the reference solution. Triplicate determinations were made for each sample.

After the initial determination, the procedure was repeated at 1 hour intervals for 6 hours and then again at 24 hours. During this period of time a sufficient amount of drug was added to maintain an excess at all times. Once the decomposition of methyclothiazide was detected at pH 8.6, 9.0 and 9.4, the regular monitoring of the concentration was abandoned and all of the suspensions were allowed to stand at room temperature for 22 days without agitation. At the end of the 22 day equilibration period the suspensions were filtered through Whatman #1 filter paper and

centrifuged. A 0.01 ml aliquot was pipetted into a 1 cm Hellma quartz cuvette and then diluted with 2.9 ml of buffer and the absorbance was then read at 267 nm with the buffer serving as the reference in a matching cuvette.

b. Bendroflumethiazide

An excess of the pure drug was shaken with 10 ml of borate buffer of the appropriate pH value and agitated continuously for 3 hours at a constant temperature of 25 ± 1°C. Borate (Sorensen) buffer has an ionic strength of 0.1 M and the pH values examined were 8.4, 9.0 and 9.6. Triplicate determinations were made for each sample.

3. Detection of Decomposition by Thin Layer Chromatography

The suspensions of methyclothiazide were monitored for decomposition at 15 minutes, 30 minutes, 45 minutes, 1 hour, 1 1/2 hours, 2 hours, 3 hours, 24 hours, and 22 days following the initial exposure of the drug to the buffer solutions, whereas suspensions of bendroflumethiazide were only monitored for 3 hours.

At the appropriate time interval a 50 µl aliquot of each sample was removed and diluted to 150 µl with acetone. The acetone-water aliquot was then applied in 10 and 30 µl quantities to a silica gel-coated microscope slide and the subsequent analysis was identical to that described in Procedure B.

D. Ultraviolet Absorption Spectra of Benzothiadiazines and Diazoxide

Solvent effects on the absorption maxima and the variation of the absorption spectra with pH were examined for each of the selected benzothiadiazines and diazoxide. The analytical wavelength for each drug was also determined.

1. Solvent Effects on the UV Absorption Maxima

Stock solutions of each drug (1×10^{-4} M) were prepared in 1% ethanol, 95% ethanol, and dimethylformamide and a 5 ml aliquot of each stock solution was then diluted to 25 ml, in a volumetric flask, with either 1 N HCl or 0.1 N KOH. This technique provided acidic and basic 2×10^{-5} M sample solutions, whereas the reference solutions contained 5 ml of the solvent (either 1% ethanol, 95% ethanol, or dimethylformamide) in place of the stock solution. Each sample was then scanned against its reference on a Unicam SP 1800 spectrophotometer. The wavelength range was 200-390 nm for the ethanolic solvents and 250-390 nm for samples containing dimethylformamide. A matched pair of 1 cm Hellma quartz cuvettes with caps was employed in the scanning procedure.

2. Variation of UV Absorption Spectra with pH

The appropriate quantity of drug was dissolved in a sufficient volume of 95% ethanol to prepare a 1×10^{-4} M stock solution. A 5 ml aliquot of each stock solution was then diluted to 25 ml with the buffer of the desired pH value and reference solutions were prepared by the substitution of 5 ml of 95% ethanol in place of the drug stock

solution. Each sample was scanned against its reference on a Beckman Model 25 spectrophotometer over the wavelength range of 200-360 nm.

This procedure was carried out for each drug at intervals of one pH unit, covering the pH range of 0-14. The pH was modified by the addition of acid, buffer or base as follows:

pH	Buffer
0	1 N HCl
1	0.1 N HCl
2	0.01 N HCl
3	0.001 N HCl
4	succinate
5	phosphate (Sorensen)
6	phosphate (Sorensen)
7	phosphate (Sorensen)
8	phosphate (Sorensen)
9	glycine/NaOH (Sorensen)
10	glycine/NaOH (Sorensen)
11	glycine/NaOH (Sorensen)
12	glycine/NaOH (Sorensen)
13	0.1 N KOH
14	1 N KOH

3. Determination of the Analytical Wavelength

Since the UV absorption spectrum at each pH value had been scanned on the same page of the recording chart, these superimposed spectra were used to determine the analytical wavelength. The wavelength at which the absorbance differences between the diprotonated, monoprotonated, and nonprotonated species were the greatest appeared to be 270 nm for all of the benzothiadiazines and diazoxide.

E. Determination of Benzothiadiazine Acidity Constants by UV Spectrophotometry

The acidic dissociation constants of diazoxide, methyclothiazide, and polythiazide, as well as the overlapping acidic ionization constants of selected benzothiadiazines, were determined by the ultraviolet spectrophotometric method.

1. Diazoxide, Methyclothiazide and Polythiazide

The acidic dissociation constants of methyclothiazide, polythiazide, and diazoxide were determined by the conventional technique described by Albert and Serjeant (20). Approximate pKa values were first obtained from the superimposed UV absorption spectra and then the exact pKa values were determined in the following manner:

a. Stock Solution

A 1×10^{-4} M stock solution of each drug was prepared in approximately 1% ethanol by first dissolving a sufficient quantity of the drug in 5 ml of 95% ethanol and then diluting the solution to 500 ml with glass distilled, deionized water free of carbon dioxide.

b. Exact pKa Determinations

A 5 ml aliquot of the appropriate 1×10^{-4} M stock solution was diluted to 25 ml while 5 ml of a 1% ethanol blank was used

in the reference solution. Seven 2 x 10^{-5} M samples, which covered the range of the approximate pKa ± 1 unit, were prepared. The pH of each solution was obtained with a Fisher Accumet 320 expanded scale pH meter at the recorded temperature and the absorbances were read at the selected analytical wavelength on a Beckman Model 25 spectrophotometer. Absorbance readings and pH values were also obtained for the nonprotonated species in 0.1 N KOH and for the monoprotonated species in an appropriate buffer. The buffers for the monoprotonated species were selected after examining the UV absorption spectra obtained in Procedure D, namely pH 5, 6 and 7 for diazoxide, methyclothiazide and polythiazide, respectively.

2. Determination of Overlapping Dissociation Constants

Generally, the initial steps in the procedure were similar to those previously described. An accurately weighed quantity of the drug was dissolved in 10 ml of 95% ethanol and a sufficient volume of glass distilled, deionized, carbon dioxide-free water was added to make the volume equal to one liter. This provided a 1 x 10^{-4} M stock solution in 1% ethanol.

From the stock solution 2 x 10^{-5} M samples were prepared at 0.2 pH unit intervals to cover the pH range between the diprotonated and the nonprotonated species. Identical reference solutions contained 5 ml of a 1% ethanol blank.

For chlorothiazide and flumethiazide, the buffers used were of 0.01 M ionic strength while the buffers for the hydrothiazides were maintained at 0.1 M ionic strength.

Thiazides		Hydrothiazides	
pH range	buffer	pH range	buffer
4.00-6.20	succinate	5.0- 7.6	phosphate (Sorensen)
6.40-7.60	phosphate	5.8- 9.2	sodium borate/HCl (Sorensen)
7.80-8.40	TRIS	9.4-10.6	sodium borate/NaOH (Sorensen)
8.60-9.60	borate	10.8-12.8	glycine/NaOH (Sorensen)
9.80-10.60	carbonate		

Buffers in the pH range of 4-7 furnished the medium for the diprotonated species and buffers in the pH range of 10.2-12.8 were adequate for the nonprotonated species. To demonstrate complete ionization the nonprotonated form was also examined in 0.1 N KOH.

Sample pH and absorbance, at the analytical wavelength of 270 nm, were read at the recorded temperature and the computer input data were selected from the linear portions of the graphical representations of absorbance versus pH.

F. Determinations of Deprotonation Order

To determine the order in which the two acidic protons of the benzothiadiazines ionize, methyclothiazide and hydrochlorothiazide, in semiaqueous solutions, were examined by NMR spectrometry.

1. Preliminary Titrations

A 10 ml sample of methyclothiazide 0.02 M was prepared by transferring 2.5 ml of a 0.12 M methyclothiazide acetone stock solution to a 50 ml beaker and then adding 4 ml of acetone and 3.5 ml of distilled water to the titration vessel. The initial pH of the sample was recorded and then 40% NaOH was added in 0.01 ml portions. After each

0.01 ml addition of the titrant the pH of the sample solution was measured and the titration was terminated at pH 13.

The titration procedure was repeated on smaller sample volumes using a 5 ml beaker as the titration vessel. A 1 ml and a 0.5 ml sample was prepared and the titrant was delivered using a 1 µl pipette with disposable pipette tips. The pH was measured with Whatman-BDH narrow range pH paper and adequate stirring of the sample solution was achieved with a capillary tube 1.6 mm in diameter.

A similar 'microtitration' was carried out for hydrochlorothiazide. A 0.0134 g quantity of the drug was weighed into a 10 ml beaker and 0.75 ml of acetone was added to the vessel. Once the drug had completely dissolved in the acetone, 0.75 ml of distilled water was added to give 1.5 ml of a 0.03 M solution. The titration and the pH measurements were performed as previously described for methyclothiazide.

2. Sample Preparation for NMR Spectrometry

All of the solvents employed in this section of the procedure were of spectral grade quality, specifically manufactured for NMR and IR spectrometry. Proton NMR spectra were obtained on a Bruker WH-200 high resolution spectrometer using the pulsed Fourier transform mode. The probe temperature was maintained at 30 ± 1°C, the thin-walled NMR tubes were 5 mm in diameter, and the internal standard was TMS.

a. Methyclothiazide

A 0.01060 g quantity of methyclothiazide was weighed into a 10 ml beaker and 1 ml of deuterated acetone was added to dissolve the drug. The addition of 0.5 ml of D_2O yielded 1.5 ml of a 0.02 M methyclothiazide solution. A 0.5 ml aliquot of this solution was transferred to an NMR tube and the sample was scanned. The sample was then brought to pH 13 by the addition, directly into the NMR tube, of 2 µl of 40% NaOD.

b. Hydrochlorothiazide

A 0.0134 g quantity of hydrochlorothiazide was weighed into a 10 ml beaker, 0.75 ml of deuterated acetone was added, and the solution was stirred until the drug had completely dissolved. The addition of 0.75 ml of D_2O brought the sample volume to 1.5 ml and the drug concentration to 0.03 M. A 0.5 ml aliquot of the solution was transferred to an NMR tube and the sample was scanned.

The remaining 1.0 ml of the sample was titrated with 1 µl of 40% NaOD and a 0.5 ml aliquot (containing 0.5 µl of titrant) of this solution was scanned. Further 1 µl additions of titrant directly into the NMR tube were followed by the rescanning of the sample until a maximum of 4 µl of titrant had been added.

Only 0.3 ml (containing 0.3 µl of titrant) of the remaining sample could be accurately delivered to the NMR tube so the volume was brought to 0.4 ml by the addition of 0.1 ml of

deuterated acetone. The titrant was added in 0.5 µl portions until approximately 3 µl had been added to the sample and an NMR spectrum was obtained following each addition of titrant. This portion of the procedure was only qualitative since the titrant was added in estimated volumes of 0.5 µl. These estimated additions of the titrant were necessary to provide additional points for the titration curve and also to observe the effect of concentration changes on the NMR spectra.

The estimated volumes of the titrant required for the 0.0225 M sample of hydrochlorothiazide were converted to the comparative volumes which would be required for a 0.03 M sample to obtain the same degree of ionization.

Volume of 40% NaOD (µl) added to hydrochlorothiazide 0.0225 M	Comparative volume of 40% NaOD (µl) required for hydrochlorothiazide 0.03 M
0.8	1.1
1.3	1.7
1.8	2.4
2.8	3.7
3.3	4.4

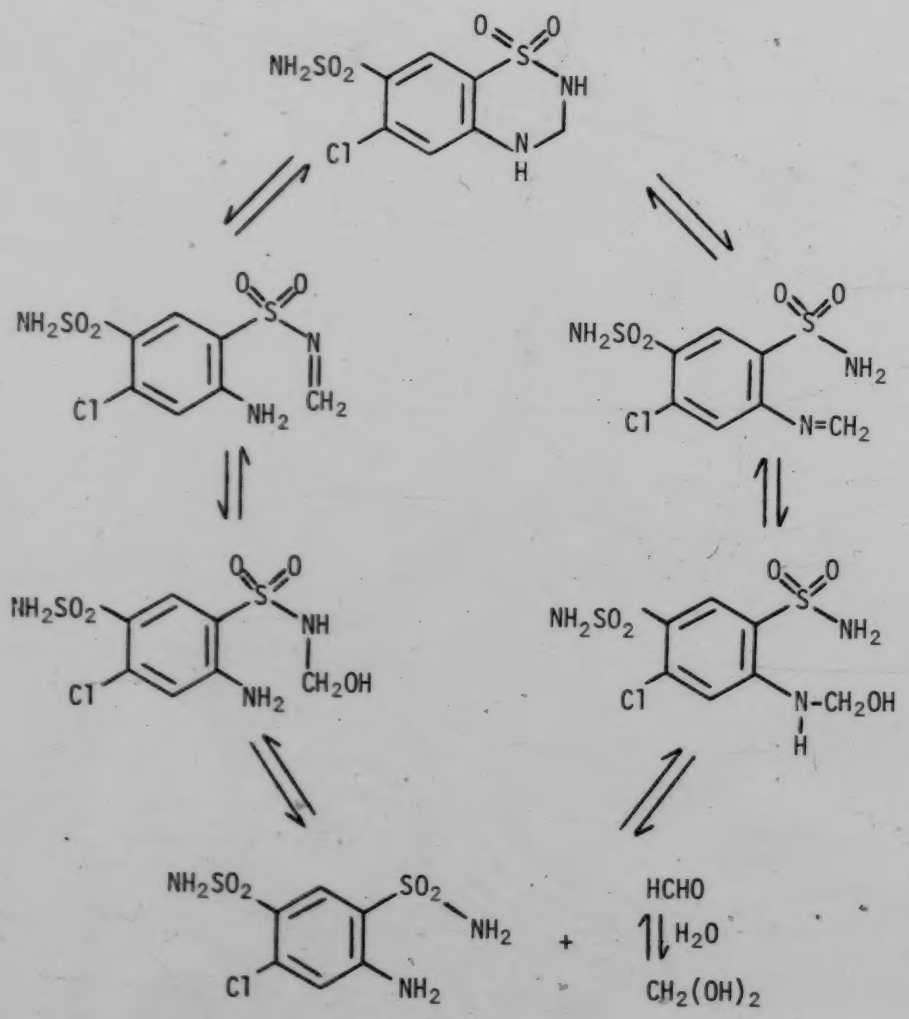
DISCUSSION AND RESULTS

The Decomposition of Benzothiadiazines

In the search for the ideal method of determining the dissociation constants of the benzothiadiazines, the utility and applicability of previously reported methods were first examined. Ågren and Bäck (18) employed the solubility variation with pH technique in determining the first acidic ionization constant of bendroflumethiazide. Their experimental conditions were such that significant decomposition would be expected but they do not appear to have taken this into account in their publication.

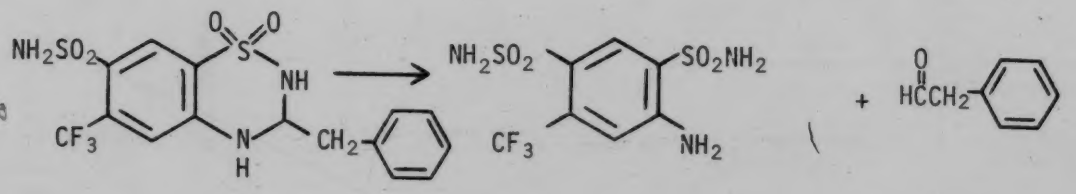
Accordingly, some experiments were designed to study the effect of pH on the stability of the benzothiadiazines. The investigation was initiated with the preparation of the three different decomposition products that could result from the various benzothiadiazines and these reference decomposition products were obtained by the alkaline hydrolysis of bendroflumethiazide, cyclothiazide, and polythiazide. Boiling with alkali readily promotes the opening of the thiadiazine ring system, as has been observed by Close et al. (5), with the formation of a benzenedisulfonamide. This type of hydrolysis reaction under basic conditions has been studied by Mollica et al. (13,83), who investigated these effects on hydrochlorothiazide at 60°C in 1 N NaOH. Hydrochlorothiazide is synthesized by the condensation of 4-amino-6-chloro-1,3-benzenedisulfonamide with formaldehyde under acidic conditions and the alkaline hydrolysis of hydrochlorothiazide results in the reformation of these initial reactants. Therefore, reversible kinetics explains the reaction.

The postulated two-step mechanism (13) involves the initial thiadiazine ring opening to form an imine which can undergo attack by hydroxide ion or water to produce a carbinolamine. The amino-alcohol then decomposes to formaldehyde and 4-amino-6-chloro-1,3-benzenedisulfonamide, as outlined by the following chemical scheme:

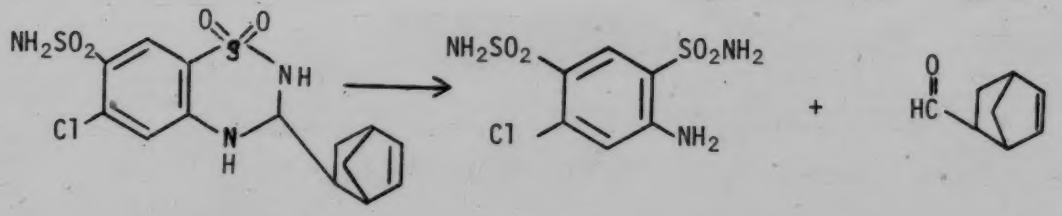


The ring opening can occur via several pathways and this makes possible the formation of an imine with the cyclic sulfonamido nitrogen or with the basic nitrogen at the 4-position, although the end result of either pathway is the formation of the original benzenedisulfonamide and formaldehyde.

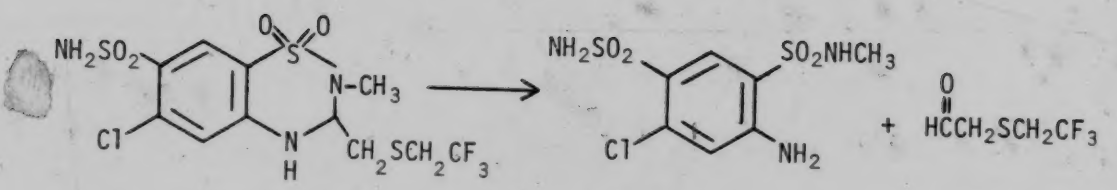
Substitution at the 3-position affects the overall rate of the reaction (13) but when 3-substituted benzothiadiazines are refluxed in 20% NaOH a complete hydrolysis, to produce the corresponding benzenedisulfonamide, can be expected. The mechanism described for the hydrolysis of hydrochlorothiazide can also apply to bendroflumethiazide, cyclothiazide, and polythiazide, leading to the desired benzenedisulfonamides (I, II and III).



bendroflumethiazide I



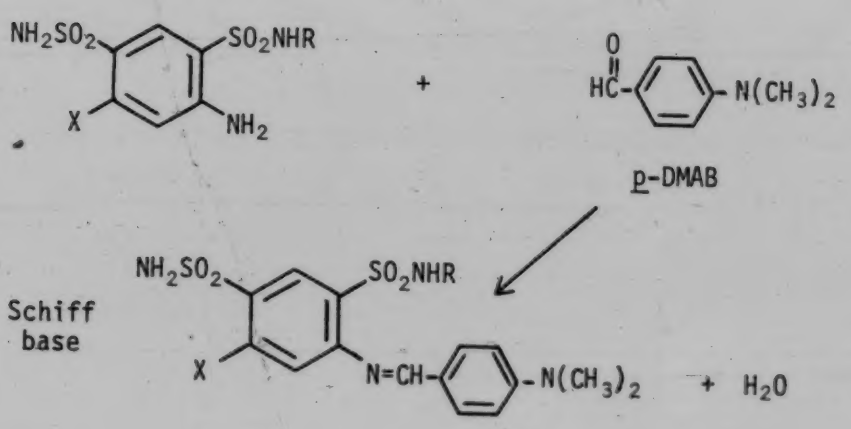
cyclothiazide II



polythiazide III

The identity of the recrystallized benzenedisulfonamides was confirmed using melting point determinations, thin layer chromatography, infrared spectra, and mass spectra.

The thin layer chromatographic analysis in an ethylacetate : benzene (8:2) solvent system provided R_f values for I, II and III and the presence of the primary aromatic amino group in the three benzenedisulfonamides was confirmed from their reaction with acidified p-DMAB spray reagent. Lemon yellow spots, which increased in color intensity as the concentration of the substrate increased, indicated the reaction of the primary aromatic amino group of each of the three benzenedisulfonamides with p-DMAB to produce a Schiff base.



p-DMAB

Schiff base

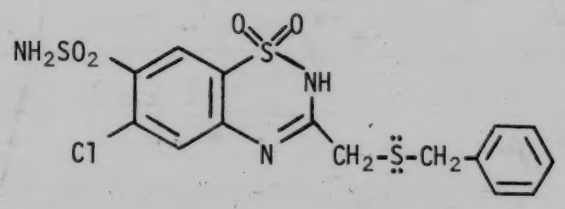
The Schiff base extends conjugation to produce a colored compound and the resulting shift to a longer absorption wavelength means that the compound now absorbs in the visible range of the spectrum.

The ultraviolet absorption spectra of the three benzenedisulfonamides are very similar to the spectra of the three parent compounds because the basic chromophore has not been altered sufficiently to effect a change in the wavelength of maximum absorption. The benzene ring, together with the two sulfonamido groups, are the major groups contributing to the conjugation and, therefore, to the wavelength of maximum absorption. The basic amino group becomes a free primary aromatic amino group and this may cause a shift of a few nanometers to longer wavelength but the shift is not significant and it does not distinguish between the three parent benzothiadiazines and their decomposition products.

In contrast, Baer et al. (12), in their study of chlorothiazide, examined the UV absorption spectrum of the decomposition product obtained in a basic medium and observed a 30 nm shift in wavelength. The hydrolysate absorbed at 261 nm while chlorothiazide, in dilute base, absorbed at 290 nm. This shift in the wavelength of maximum absorption is significant and hence a UV absorption spectrum can provide a reliable indication of the presence of the decomposition product of chlorothiazide.

Ultraviolet absorption spectra would provide similar information for flumethiazide and benzthiazide. The decomposition product of benzthiazide is identical to that of chlorothiazide and cyclothiazide and the decomposition products of flumethiazide and bendroflumethiazide

are identical. Thiazides (chlorothiazide, flumethiazide, and benzthiazide) are 3,4-unsaturated benzothiadiazines and this double bond extends conjugation, resulting in maxima at 292 nm in 0.1 N KOH and 282 nm in 0.1 N HCl for chlorothiazide. Flumethiazide has a trifluoromethyl group at the 6-position and, therefore, it exhibits broad peaks at 280 nm in dilute base and 270 nm in dilute acid. The 3-substituent of benzthiazide is a chromophore in itself and contains a sulfur atom which causes a shift in the absorption to longer wavelength.



benzthiazide

The structure of the 3-substituent readily explains the absorption maxima of benzthiazide at 292 and 315 nm in dilute KOH and at 280 nm in dilute HCl. Once the ring opening has occurred the double bond disappears, reducing the extent of the conjugation and the decomposition product absorbs at the significantly shorter wavelength of approximately 270 nm.

Hydrothiazides, such as bendroflumethiazide and cyclothiazide, and N^2-methylhydrothiazides, such as polythiazide, are 3,4-saturated benzothiadiazines and the reduction of this double bond limits the extent of conjugation. For these benzothiadiazines, the UV absorption maxima occur at approximately 270 nm in both dilute acid and base, a

shorter wavelength than for the thiazides, and their decomposition products (benzenedisulfonamides I and II, and benzenesulfonamide III) also absorb at this wavelength. Since the hydrothiazides and their decomposition products have similar UV absorption spectra, the use of ultraviolet spectrophotometry to quantitatively measure the drug content of samples in the solubility variation with pH technique will not permit the detection of any decomposition that is taking place in the basic buffers. The pKa value ultimately derived from the solubility data may be that of the decomposition product or, more likely, a mixture of the parent hydrothiazide and its hydrolysis product.

The occurrence of decomposition during the solubility studies was evident from the TLC analysis of the solutions, which was performed at various time intervals during the procedure. The TLC analysis revealed the presence of the decomposition products of bendroflumethiazide and methyclothiazide. The use of GLC or HPLC, rather than UV spectrophotometry, to quantitate the drug content of the solubility sample solutions would be advantageous since these detection methods make possible the monitoring of not only the drug concentration, but also any decomposition products.

Samples of methyclothiazide and bendroflumethiazide in buffers higher than pH 9, obtained from the solubility studies, were analyzed by TLC. The results of the analysis indicated the presence of the benzothiadiazines and their decomposition products, as was confirmed by the R_f values and by the reaction with acidified p-DMAB spray reagent (84-86). After the application of the acidified p-DMAB, violet purple spots appeared at the same R_f values as methyclothiazide

and bendroflumethiazide and these violet purple spots exhibited increased color intensity as the pH values of the buffers were increased. The aqueous samples from the solubility studies were diluted with acetone before they were spotted onto the TLC plates, whereas bendroflumethiazide and methyclothiazide were dissolved in ethylacetate prior to TLC analysis. Since the violet purple color was not produced with samples from the ethylacetate stock solutions, the acidified p-DMAB must react with the benzothiadiazines only when they are in their ionized form. This is demonstrated by the fact that the color only appears when buffers higher than pH 9 are used in the solubility studies, and this is in the pH region following the first acidic pKa value. The purple color fades soon after its development.

The type of colored complex that is formed between the benzothiadiazines and p-DMAB is difficult to postulate since there is no apparent record of Schiff base formation with the nitrogen of a sulfonamido group. Schiff base formation with primary aromatic and aliphatic amines yields yellow, orange-red, or brown colored products but a violet color occurs when pyrrole reacts with p-DMAB (37), and this product rearranges into a quinoidal compound.

The functional group of the benzothiadiazines that most closely resembles a primary amino group is the exocyclic sulfonamido group and the rapid fading of the color indicates an unstable complex which dissociates soon after formation. The conditions for Schiff base formation with the exocyclic sulfonamido group are not ideal and this may explain the rapid dissipation of the purple color. In addition,

it is noted that the colored complex only appears when a salt form of the benzothiadiazine has been achieved and this may confer some stability on the compound, thus allowing the momentary complex formation. The transient complex probably extends conjugation and, consequently, shifts the wavelength of maximum absorption into the visible range of the spectrum.

The colored complexes that have been observed in TLC analysis transmit yellow and purple light, therefore, they absorb blue and yellowish-green light. Their wavelengths of maximum absorption are 458 and 570 nm, respectively (88).

The formation of the decomposition products has served a useful purpose in the quantitative analysis for the benzothiadiazine content of dosage forms and biological fluids. First, the benzothiadiazine was hydrolyzed in an alkaline medium at boiling temperatures to produce a benzenedisulfonamide, then, a colored azo compound was formed through the primary aromatic amino group of the benzenedisulfonamide. The diazotization and coupling of the aromatic amine was achieved by the Bratton-Marshall method (89) and the colored product was analyzed at 518 nm. This type of a procedure was used in the quantitative analysis for chlorothiazide bioavailability studies (15) and in the determination of chlorothiazide levels in plasma and urine (12).

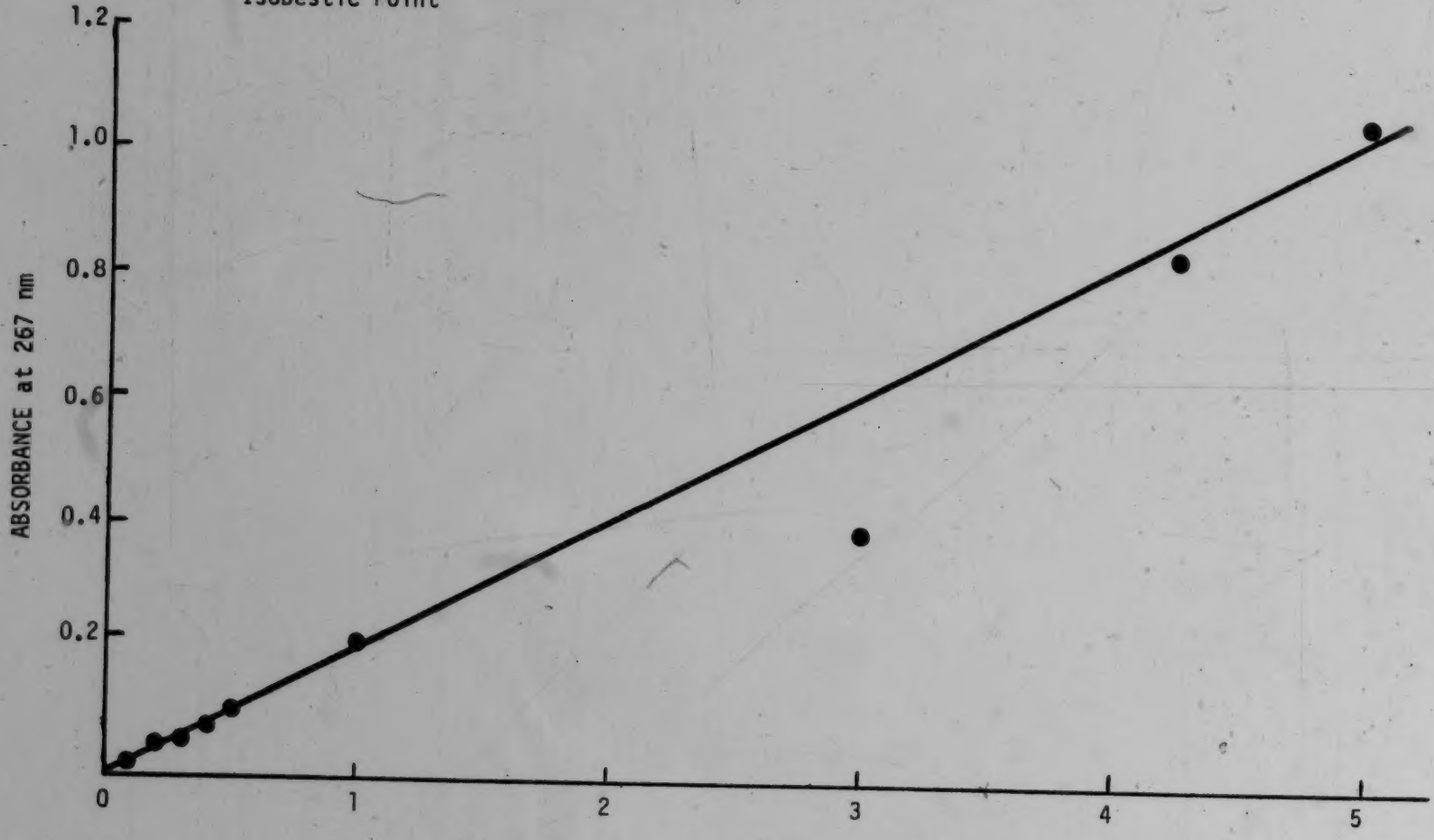
Solubility Studies

The presence of the decomposition product in the sample solutions from the solubility studies is not desirable. The solubility variation with pH method of determining acidity constants was carried out with methyclothiazide and bendroflumethiazide to determine the extent and significance of decomposition in buffers of high pH.

For methyclothiazide, the standard curves at each pH were determined in 20% ethanolic solutions to ensure the complete solubility of the drug and it was found that one concentration curve, prepared at the isobestic point, was suitable for the solubility studies (Figure 1). The isobestic point is independent of pH and the absorbance, therefore, is only affected by changes in the concentration and this is evident from the standards at each individual pH. The ethanolic content and the incremental pH changes of 0.4 pH units showed a minimal effect on the absorbances of the standard, and on the isobestic point of methyclothiazide at 267 nm. Table 3 compares the calibration curve data at each pH level to those at the isobestic point.

The monitoring of the methyclothiazide solubility sample solutions by TLC indicated that at the pH values of 8.6, 9.0 and 9.4 a detectable degree of decomposition had occurred after one hour of agitation. A second spot was visible under short wave (254 nm) ultraviolet light and this spot had the same R_f value as the corresponding reference decomposition product (benzenesulfonamide III). The spot did not visibly react with acidified p-DMAB because of the low concentration of the decomposition product present in the sample at that point in

*Figure 1. Calibration Curve for Methyclothiazide in Solubility Studies Determined at the Isobestic Point



* The point at 3×10^{-5} M may be due to experimental error but since this calibration curve was not used for quantitative purposes, the absorbance at this concentration was not redetermined.

Table 3: UV Absorbance Readings of Methyclothiazide at Selected pH Values

Concentration (moles/l)	pH 8.6	9.0	9.4	9.8	10.2	10.6	11.0	Isobestic Point Absorbances * (Determined at pH 8.6)
5×10^{-5}								1.051
4×10^{-5}								0.836
3×10^{-5}								0.401
1×10^{-5}	0.203	0.193	0.200	0.214	0.220	0.225	0.201	0.217
8×10^{-6}	0.168	0.155	0.160	0.148	0.152	0.170	0.183	
6×10^{-6}	0.128	0.111	0.123	0.113	0.111	0.123	0.118	
5×10^{-6}								0.106
4×10^{-6}								0.080
3×10^{-6}								0.063
2×10^{-6}	0.045	0.040	0.035	0.030	0.039	0.040	0.049	0.055
1×10^{-6}	0.026	0.026	0.021	0.016	0.025	0.022	0.018	0.020
8×10^{-7}	0.020	0.023	0.023	0.011	0.022	0.008	0.014	
6×10^{-7}	0.012	0.013	0.008	0.009	0.020	0.006	0.008	
5×10^{-7}								0.008
4×10^{-7}	0.006	0.002	0.006	0.008	0.007	0.004	0.007	
2×10^{-7}	0.004	0.001	0.004	0.002	0.002	0.000	0.000	
1×10^{-7}	0.001	0.000	0.000	0.001	0.000	0.000	0.001	
λ of maximum absorption	267	267	267	265	264	264	264	267

* These standards were prepared from a different methyclothiazide stock solution. Discrepancies are due to the fact that the 8 calibration curves were not obtained under identical experimental conditions.

time. The color intensity increased as the time of exposure of the benzothiadiazine to the basic buffers was extended.

At pH 8.6, after one hour of exposure to the buffer, the degradation product was only visible under short wave UV light but after 3 hours of exposure a faint yellow spot appeared after the application of acidified p-DMAB. The spot increased in color intensity after further exposure of the drug to basic buffers. Triplicate samples were examined at each pH level and decomposition was observed before equilibration had been achieved. Equilibration is not complete until any further change in the drug concentration is less than 3%, as determined by a suitable method of analysis. The three hour limit of agitation time set by Green (70) and used by Ågren and Bäck (18) was not adequate for bendroflumethiazide or for methyclothiazide, as can be deduced from Table 4, since the drug concentration was still changing once decomposition had started. After 22 days of equilibration the drug content of each of the three samples was the same but decomposition was evident from the intensely yellow spots that appeared upon TLC analysis.

The observed decomposition obviously precludes the use of the solubility method, as it was described by Green (70) and Peck and Benet (75), for the benzothiadiazines since the length of the agitation period and the basic buffer conditions required for the equilibration are not ideal for achieving equilibration before decomposition begins. Since the thiadiazine ring opening under basic conditions at a pH higher than 8 (12,90,91) is common to the benzothiadiazines, the experimental conditions required for the solubility studies would

Table 4. Absorbance Changes for Methyclothiazide Relative to Equilibration-Agitation Periods

Time (hours)*	pH	Absorbance at 267 nm #1	#2	#3
0.5	8.6	1.138	1.146	1.198
1.0		1.220	1.231	1.277
3.0		1.277	1.298	1.338
4.0		1.297	1.318	1.354
5.0		1.303	1.320	1.372
6.0		1.337	1.343	1.390
24.0		1.410	1.416	1.440
528.0		0.105	0.105	0.110
1.0	9.0	0.938	0.842	0.893
2.0		1.152	1.040	1.088
3.0		1.263	1.133	1.171
4.0		1.346	1.239	1.254
5.0		1.412	1.333	1.324
6.0		1.400	1.393	1.435
24.0		1.516	1.571	1.597
26.0		1.711	1.715	1.697
28.0		1.790	1.808	1.745
528.0		0.175	0.202	0.178
3.0	9.4	1.799	1.850	1.890
528.0		0.243	0.280	0.232

* The absorbance values at 22 days of equilibration are for diluted samples (0.1 ml of filtrate plus 2.9 ml of the appropriate buffer).

readily promote this decomposition. Equilibration may be reached more rapidly in buffers of high pH because the benzothiadiazines are more soluble in basic media, but decomposition also occurs more readily in basic media.

Methods of Determining Benzothiadiazine Acidity Constants

The other methods available for determining the dissociation constants of the benzothiadiazines are potentiometry (both aqueous and semiaqueous), ultraviolet spectrophotometry, and distribution studies. The most convenient method is aqueous potentiometry and a few of the benzothiadiazines have been analyzed by this technique (7, 13,19). The compounds examined were soluble at 0.001 M concentrations in water but most of the benzothiadiazines are sparingly soluble in water and a nonaqueous solvent must be added to ensure that the compounds remain in solution. This would suggest semiaqueous potentiometry as a logical choice for investigating acidity constants but the method is only useful as a comparative means and the data obtained may not represent a true aqueous acidity value.

The second acidic pKa appears to be especially affected by dimethylformamide, as has been observed by Whitehead et al. (7). Chlorothiazide, when titrated in water, gave pKa values of 6.8 and 9.4 but when titrated in 66% dimethylformamide the values were 6.9 and 12.1. The high second pKa value has been attributed to the solvation of the exocyclic sulfonamido group. Substitution at the 3-position, while leaving the 3,4-double bond intact, resulted in significant variations

of the first acidic pKa, which ranged from 5.4-8.2 (7). On the other hand, the second pKa did not exhibit any variation at all but remained within a range of 12.0-12.5 with an average of approximately 12.2. These are excellent comparative values for the first acidic group but they cannot be directly related to those values obtained in aqueous media. For comparative values each benzothiadiazine must be sufficiently soluble in the chosen solvent to allow titration and it is not always possible to find a suitable solvent that is water miscible in all proportions.

The extrapolation technique was not a viable choice since sparingly soluble benzothiadiazines would not be sufficiently soluble in mixed solvents of low organic solvent content to permit a reliable extrapolation to 100% water. The method, if it must be employed, may have some merit if the lowest possible organic solvent concentration is used and a very concentrated titrant is added. This would keep the volume of water reasonably constant and thus prevent the precipitation of the compound from solution.

The remaining available choices for determining the ionization constants of benzothiadiazines were ultraviolet spectrophotometry and the distribution method. Distribution studies may be ideal for compounds such as the benzothiadiazines because the method overcomes decomposition and solubility problems. A small amount of drug can be added to the solvent and the ionic strength of the buffers can be kept constant so that the concentration of drug will not affect the ionic strength of the sample. The agitation and equilibration time can be controlled to obtain relative values for the particular experimental

conditions. Only one analysis for drug content in the sample needs to be performed, rather than constant monitoring until no further change in concentration is observed. Analysis for decomposition and concentration can be achieved simultaneously by examining both the aqueous and the organic phases by a suitable detection method such as HPLC or GLC.

Two of the limitations of the distribution method deemed it to be inappropriate for investigating the acidity constants of the benzothiadiazines. Firstly, it was difficult to find an organic solvent that was immiscible with water yet would also solubilize all of the compounds of interest. It is desirable to use the same solvent for all of the compounds as comparative values cannot be obtained otherwise. Secondly, the distribution method could not provide any information regarding the order of deprotonation of the acidic protons. Thermodynamic pKa values are much more easily determined from UV spectral data and the spectra may also provide an indication of the order of deprotonation. For comparative values, all of the benzothiadiazines should be examined by the same method and preliminary studies revealed that ultraviolet spectrophotometry was the technique of choice.

In view of the fact that benzothiadiazines are sparingly soluble in water, the addition of some water miscible organic solvent which is, preferably, transparent to UV light would be required to completely solubilize these drugs. The initial step would be to examine the effect of the organic solvent on the UV spectra. Comparisons of 0.2% and 20% v/v ethanolic solutions showed no significant change in the wavelength of maximum absorption (anything less than 5 nm was considered to be insignificant (92) and a small shift in wavelength could be due to

a shift of the recorder chart paper since all other experimental conditions, such as temperature and the time of the experiment, were kept constant. The 20% dimethylformamide content did not appear to shift the wavelength of maximum absorption from approximately 270 nm in either acidic or basic media but this solvent is not transparent below 250 nm because of the presence of a carbonyl and a tertiary nitrogen which absorb light in the UV region.

The insignificant effect of ethanol on the UV spectra permitted the use of 20% ethanolic solutions to determine all of the UV spectra at 1 pH unit intervals over the pH range of 0-14 for the selected benzothiadiazines. This spectral data was also used to select the analytical wavelength and the approximate pH regions where the ionizations of the compounds occur.

UV Absorption Spectra of Benzothiadiazines

A brief review of UV spectral-structure relationships, as an introduction to the UV spectra of the benzothiadiazines, is in order. The accepted theory, as summarized by Dyer (93), involves electronic transitions caused by the absorption of UV light. Irradiation of a molecule with the appropriate wavelength, providing the correct quanta of energy, excites valence electrons and promotes them from sigma-, pi-, and n-orbitals in the ground state to higher energy states. The movements of electrons to higher energy levels are called transitions and the higher energy state is termed a star orbital.

The types of existing electrons are sigma-, pi-, and n-electrons and their excited states are sigma star, pi star, and n star orbitals.

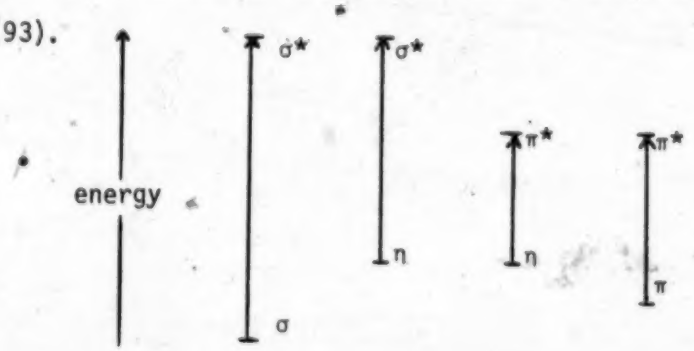
The excitation of each of these electrons requires a different amount of energy, which is supplied by the commonly used deuterium lamp, and the quanta of energy impinging upon the compound depend upon the wavelength selected.

Sigma electrons form part of a stable bond and, therefore, require the greatest amount of energy to become excited. Molecules consisting of sigma bonds absorb at very short wavelengths and these absorptions are not observed in the practical UV range of 200-340 nm. The absorption of UV light by such compounds can be seen in the vacuum UV region, below 200 nm, where light absorbing oxygen has been flushed out with nitrogen.

Less energy is required to excite n-electrons, which are more loosely bound. These electrons complete the outer orbitals of hetero-atoms (94) and compounds with atoms like O, N, S or halogens, which have nonbonding electrons, usually absorb in the practical UV range.

Pi electrons are associated with double and triple bonds and are free to move over the atomic centers of the constituent atoms. These electrons require the least amount of energy to be promoted to the next excited orbital so compounds with unsaturated centers absorb UV light of longer wavelength.

The probability of excitation and the quantity of energy required to elevate an electron to its excited state is diagrammatically displayed below (93).



The nomenclature used in UV spectrophotometry consists of some historical terms that have not, as yet, become obsolete. The term chromophore relates to the visible part of the spectrum of electromagnetic radiation and describes a group of atoms or a functional group that imparts color to a molecule. The expression was then extended to describe a functional group that is not in conjugation with any other group but exhibits a characteristic absorption in the UV or visible range.

Auxochromes are functional groups that do not absorb in the UV or visible region but when they are attached to a chromophore a shift of the absorption to longer wavelength, and an increase in the intensity of the absorption peak, is usually observed. Examples of auxochromes are n-electron containing groups such as amino, hydroxyl, sulfhydryl (and their derivatives), and the halogens.

A unique chromophore is the benzene molecule which is surrounded by two 'clouds' of pi electrons and conjecture as to its absorption spectrum certainly suggests a $\pi \rightarrow \pi^*$ transition with an intense absorption in the practical UV range. Peaks at 184, 203 and 255 nm are observed for benzene and all are associated with $\pi \rightarrow \pi^*$ transitions.

Since the vast majority of medicinal compounds contain at least one benzene ring, a further analysis of the absorption peaks is of importance. The 203 nm peak is also called the 200 band, K-band, E-band, and 1° band and the 255 nm peak has been termed the 260 band, B-band, and 2° band. These two peaks appear in the practical UV range and are characteristic of benzenoid compounds. The peaks at 184 and 203 nm have also been attributed to dipolar forms of benzene that result from

an electron transfer type of transition which occurs when the molecule is disrupted by high energy irradiation at a short wavelength.

In summary, the result of electronic transitions is a redistribution of electrons within a molecule and protonation or deprotonation creates a change in the electronic structure of the molecule, which is a chromophoric change represented by a shift in the absorption spectrum of the compound.

Benzothiadiazines are tetrasubstituted benzenes with sulfonamido ($-SO_2NH_2$), halogen (-X), amino (-N-), and sulfamyl ($-SO_2$-NH-) auxochromic groups which should shift the primary and secondary bands of benzene to longer wavelength. This does, in fact, occur since the majority of benzothiadiazines absorb at approximately 230 and 270 nm and the shoulder at about 310 nm is due to $n \rightarrow \pi^*$ transitions. The effects of individual substituents on the benzene ring are listed below (93).

benzene substituent	primary band		secondary band	
	λmax	ϵmax	λmax	ϵmax
-H	203.5	7400	254	204
-Cl	209.5	7400	263.5	190
$-SO_2NH_2$	217.5	9700	264.5	740
$-NH_2$	230	8600	280	1430

The electron donating halogen and amino group and the electron attracting sulfonamido substituent cause a bathochromic shift of the primary and secondary bands of benzene and they are responsible for this effect in the benzothiadiazines. The benzothiadiazines can be

subdivided into three categories: thiazides, hydrothiazides, and N^2-methylhydrothiazides and their spectra should exhibit characteristic absorptions that will identify their structural differences.

Thiazides have a 3,4-double bond while hydrothiazides and N^2-methylhydrothiazides are saturated at this position. The unsaturation extends conjugation and the molecule absorbs at a longer wavelength just as diazoxide, which closely resembles the thiazides, absorbs at a lower energy. The UV spectra of the hydrothiazides and the N^2-methylhydrothiazides are similar but distinctly different from the spectra of the thiazides.

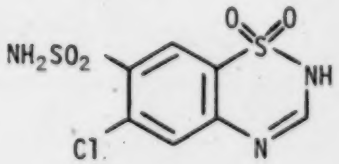
The ionization of the acidic sulfonamido protons can be traced by examining the UV absorption spectra at intervals of one pH unit. If the benzothiadiazine has only one acidic pKa only one isobestic point is observed. The degree of wavelength shift that occurs with ionization is dependent upon the electronic effects of the substituents at the 3-position, as this is the region of benzothiadiazine structural differences. The most prominent changes in wavelength and/or intensity of absorption are in the pH range where the first acidic deprotonation occurs.

When the 3-substituents are structurally similar, as the cyclic groups in cyclothiazide and cyclopenthiazide, there is an obvious similarity between the UV spectra. Many of the substituents are, in themselves, chromophores and they may also contain n-electrons which ultimately have an effect on the UV spectra. The benzyl group within the benzthiazide molecule shifts the absorption to longer wavelength and this shift is augmented by a sulfur atom. As another example,

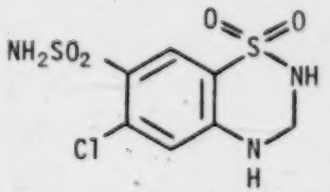
bendroflumethiazide is also substituted with this strong chromophoric benzyl group. Weak chromophores such as vinyl (-CH=CH_2) and norbornylenyl( ) are the 3-substituents of althiazide and cyclothiazide, whereas halogen atoms with n-electrons are contained in the 3-substituents of trichloromethiazide, methyclothiazide, and polythiazide.

The substituents at the 3-position should, generally, impart electronic effects onto the molecule and these effects should also affect the nearest ionizing group which is the acidic proton on the cyclic sulfonamido group. Theoretically, the effect on the UV spectrum should also explain the order of deprotonation. Chlorothiazide (Figures 5-7), at moderately acidic conditions, exhibits a maximum absorption at 280 nm and the isobestic point shifts, indicating the end of one ionization process and the start of another, as the pH is raised. With increasing pH, the wavelength shifts to lower energy until the acidic groups are completely ionized. In strongly alkaline conditions, both acidic groups are deprotonated and the wavelength of maximum absorption is 291 nm with a shoulder at 310 nm.

The extended conjugation of chlorothiazide, as compared to the conjugation of hydrochlorothiazide, can be utilized to hypothesize the order of deprotonation of the acidic sulfonamido hydrogens.

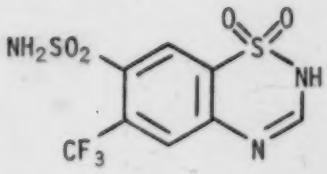


chlorothiazide

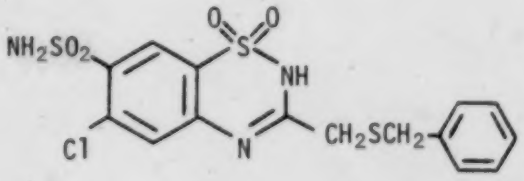


hydrochlorothiazide

The deprotonation at the 2-position is apt to alter the electronic distribution of chlorothiazide more than that of hydrochlorothiazide, as this hydrogen is attached to a heteroatom that extends the conjugation over one more atomic center in chlorothiazide. The deprotonation of the exocyclic sulfonamido group should have a minor effect on the electronic structure of chlorothiazide since that hydrogen is not involved in a direct extension of the conjugated system. The corresponding hydrogen in hydrochlorothiazide is in essentially the same chemical environment, thus supporting the theory that it is the least acidic of the protons.



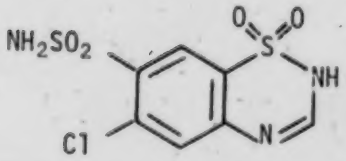
flumethiazide



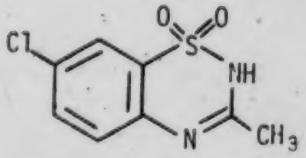
benzthiazide

Flumethiazide and benzthiazide follow the same type of spectral changes as chlorothiazide but further evidence of the deprotonation order is provided by diazoxide, which closely resembles the thiazides, both structurally and spectrally. Very little change in the UV spectra of diazoxide is observed in the pH range of 11-14 (Figure 4). The

wavelength of maximum absorption is approximately 280 nm, a shorter wavelength than for chlorothiazide, because the replacement of the exocyclic sulfonamido group with a halogen reduces the extent of conjugation.



chlorothiazide



diazoxide

The shoulder at about 310 nm is also evident in diazoxide and it is due to $\eta \rightarrow \pi^*$ transitions. In the UV spectra of the hydrothiazides and the N^2-methylhydrothiazides this shoulder becomes a peak of low intensity which appears to be due to an isolated chromophore. This effect is characteristic of the diminished $\eta \rightarrow \pi^*$ transition of the nitrogen at the 4-position when the 3,4-double bond is reduced.

The UV spectral effects caused by the deprotonation of diazoxide are similar to those caused by the ionization of the first acidic group of chlorothiazide, namely the proton at the 2-position.

The N^2-methylhydrothiazides, methyclothiazide and polythiazide, possess the basic group and the exocyclic sulfonamido group. Their UV spectra are similar to those of the hydrothiazides but only one wavelength shift occurs because only one acidic ionization is taking

place and the minor spectral changes are due to the electronic effect of this deprotonation. Both methyclothiazide (Figures 33-35) and polythiazide (Figures 36-38) exhibit an isobestic point but in alkaline solutions an unusual effect on the spectra is evident. The isobestic point is obeyed but a medium effect or decomposition (21) may be responsible for the significantly different absorptivities of these compounds at pH values of 13-14. The effect cannot be attributed to an ionization process as a second acidic ionizable group is not present in either of these benzothiadiazines.

The predictable UV spectra of the hydrothiazides exhibit two isobestic points which usually are ascribed to the ionization of the acidic groups. The ionization of the basic group is often characterized by an increase in the intensity of absorption, rather than a shift in the wavelength, but the ionization of the first acidic group results in a definite shift to a longer wavelength.

The spectra of hydrochlorothiazide (Figures 14,15) and hydroflumethiazide (Figures 16-18) are indistinguishable so the two different halogens have similar effects on the electronic structure within the molecule. Substitution at the 3-position brings about changes in conjugation and characteristic UV spectra are often produced. The addition of these substituents usually enhances or extends the chromophore, resulting in a shift to a longer wavelength. The electron attracting or donating effects of the substituents also affect the strength of the basic group and of the acidic cyclic sulfonamido group.

Trichloromethiazide (Figures 31,32) is an example of a hydrothiazide and its UV spectral characteristics can be described in relation to pH.

As the pH increases, a shift to longer wavelength, along with a decrease in absorptivity, is evident until the ionization of the second acidic group begins. Then the intensity of absorption increases and the wavelength of maximum absorption shifts to higher energy. The chlorine atoms of the 3-substituent also have an effect on the electron distribution and, therefore, on the UV spectrum of the molecule.

The other hydrothiazides follow a similar pattern of wavelength shift with the protonation and deprotonation of the basic and acidic groups. The greatest spectral change always occurs upon the deprotonation of the first acidic group.

UV SPECTRAL DATA (Figures 2-38)

The peak heights of all the spectra, except those in Figures 19, 20, 33 and 34, are an average of 25-30% higher than the literature values (12,76,77,79,92) but the positions of the maxima and minima closely correspond to the reported wavelengths. These unusually high molar absorptivities are due to a misalignment of the recorder to the spectrophotometer, which resulted in an increased spectrally recorded absorbance without affecting the digital absorbance output of the spectrophotometer.

This calibration error does not affect the qualitative value of the UV spectra and the only major distortions evident are in Figure 18 for hydroflumethiazide, Figures 21 and 22 for bendroflumethiazide, and Figure 35 for methyclothiazide. These spectra were obtained under different experimental conditions (i.e. varying temperature, a different stock solution, and/or a different calibration of the

spectrophotometer and recorder) and, therefore, these spectra do not correspond in all proportions to the other spectra of the respective compounds.

Although the distortions are present, the spectra are arranged in a manner which does not require the superimposing of one figure onto another and each figure is to be examined as a separate entity. The spectra cannot and should not be used for quantitative purposes and their qualitative aspect, as a means for comparison, is emphasized.

The figures containing these spectral distortions have been labeled with an asterisk (*) to facilitate identification. Any other discrepancies between the spectra are due to commonly encountered instrumental errors such as the recording of spectra on more than one chart paper, shifts in the chart paper during the course of the scan, filter changes within the spectrophotometer, and the shift of the baseline during the course of the scan.

UV SPECTRAL DATA

(Figures 2 - 38)

Figure 2. Absorption Spectra of Diazoxide 2 x 10^{-5} M at Various pH Values.

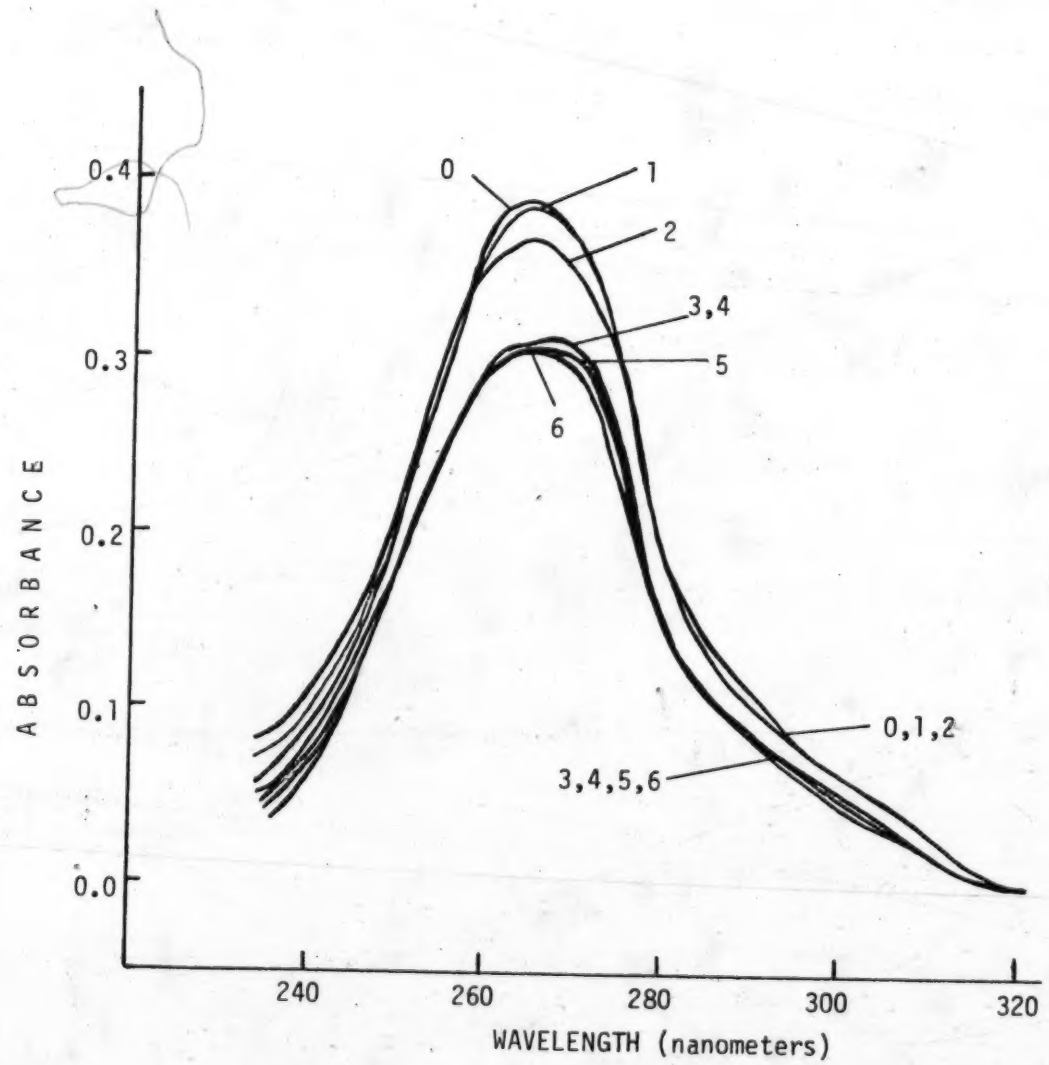
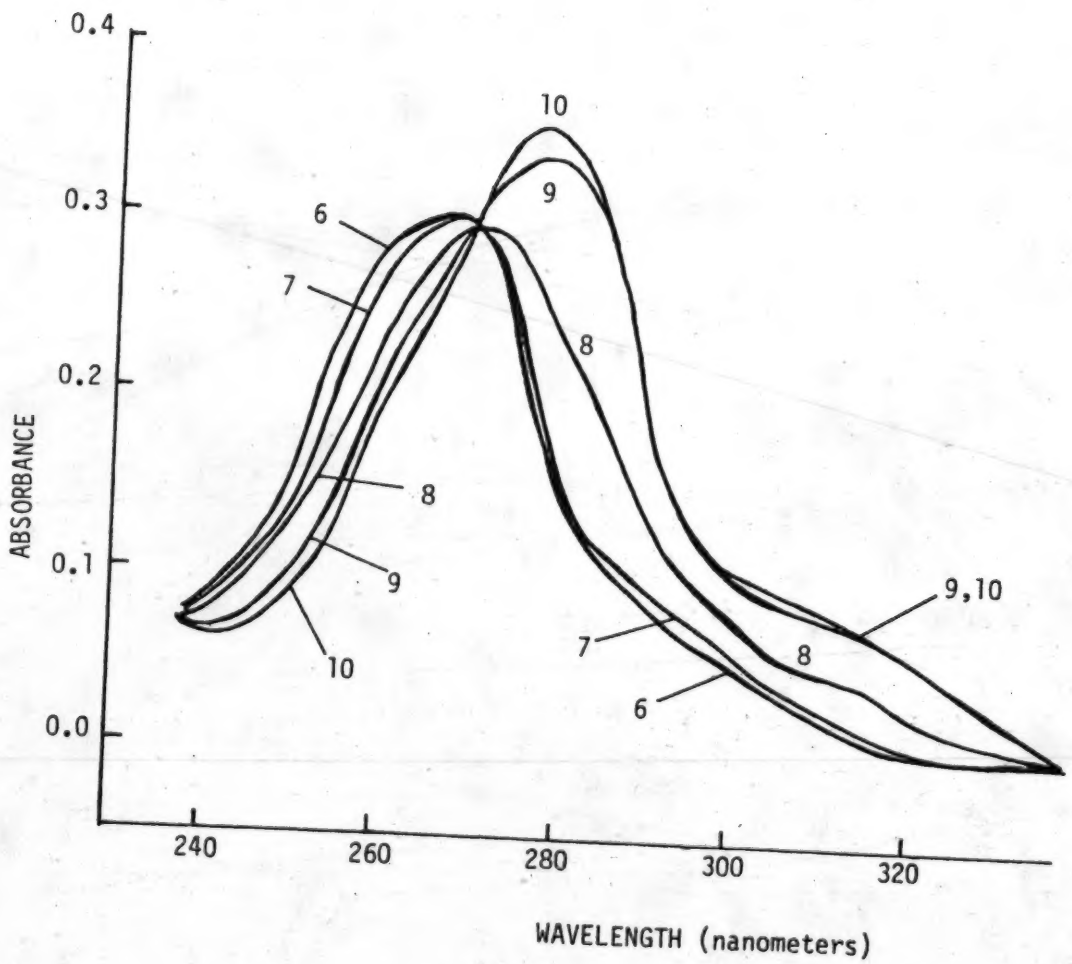
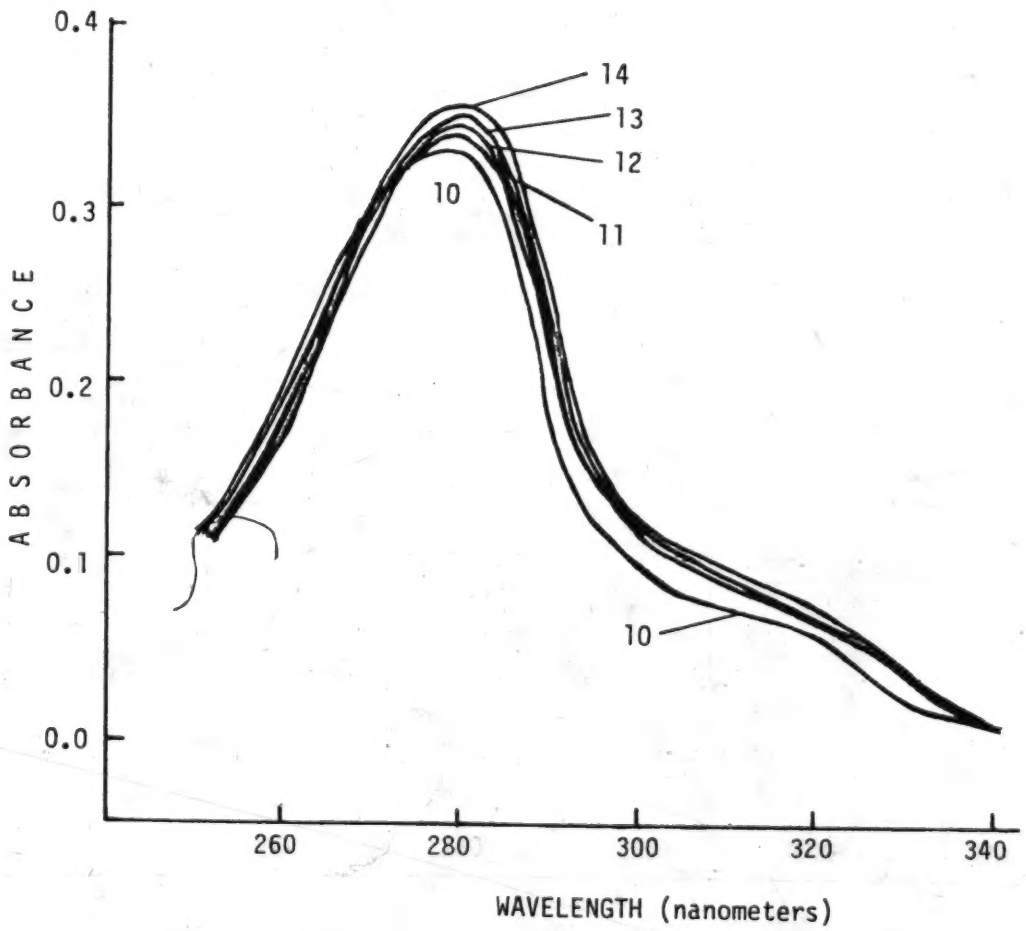


Figure 3. Absorption Spectra of Diazoxide 2 x 10^{-5} M at Various pH Values



* Figure 4. Absorption Spectra of Diazoxide 2 x 10^{-5} M at Various pH Values



* Obtained under different experimental conditions than the spectra in Figures 2 and 3.

Figure 5. Absorption Spectra of Chlorothiazide 2×10^{-5} M at Various pH Values

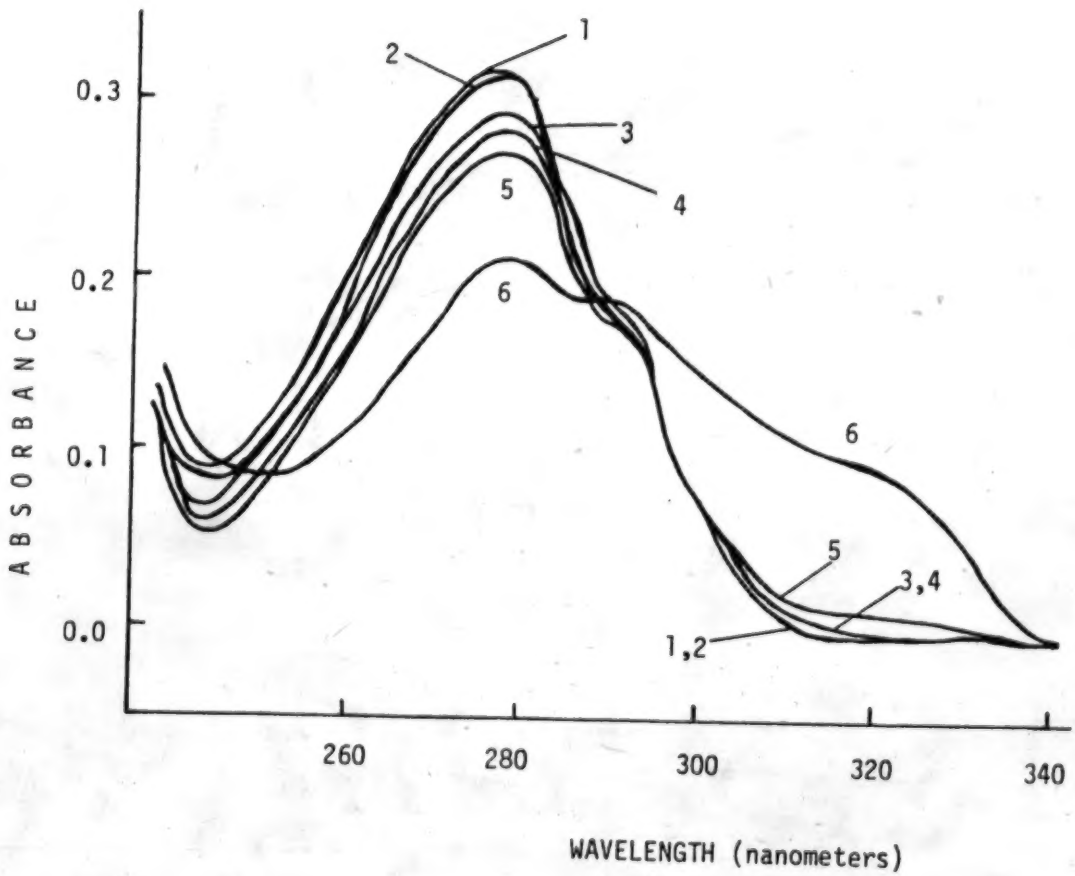


Figure 6. Absorption Spectra of Chlorothiazide 2×10^{-5} M at Various pH Values

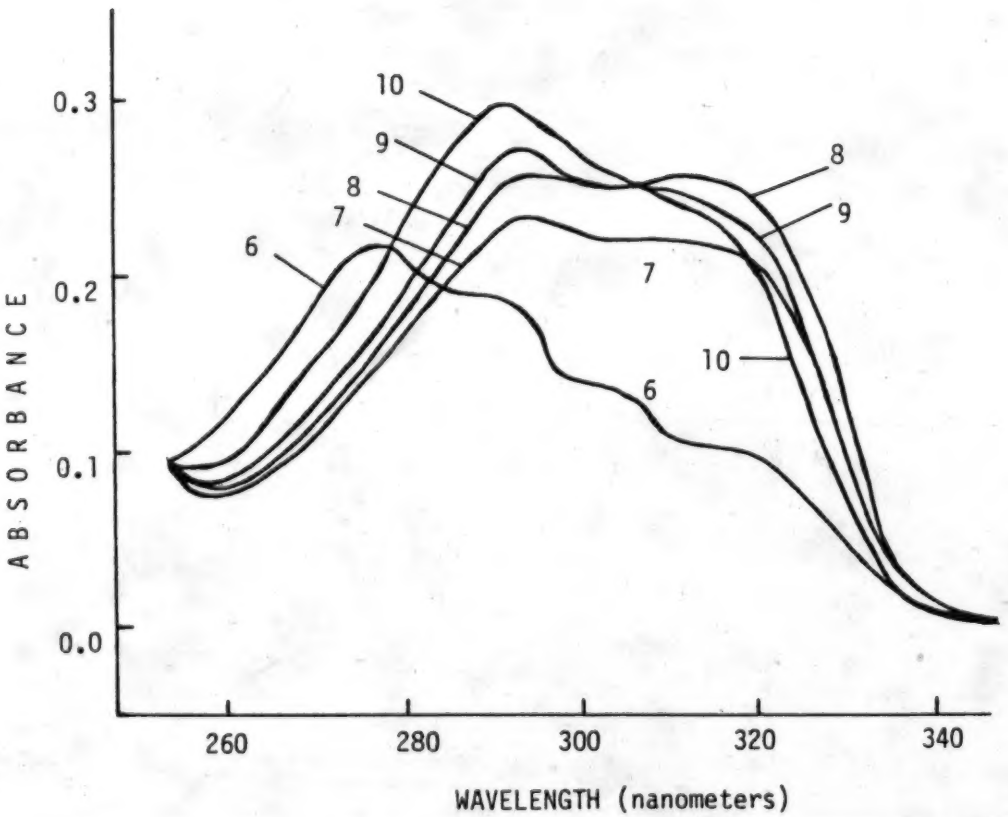


Figure 7. Absorption Spectra of Chlorothiazide 2×10^{-5} M at Various pH Values

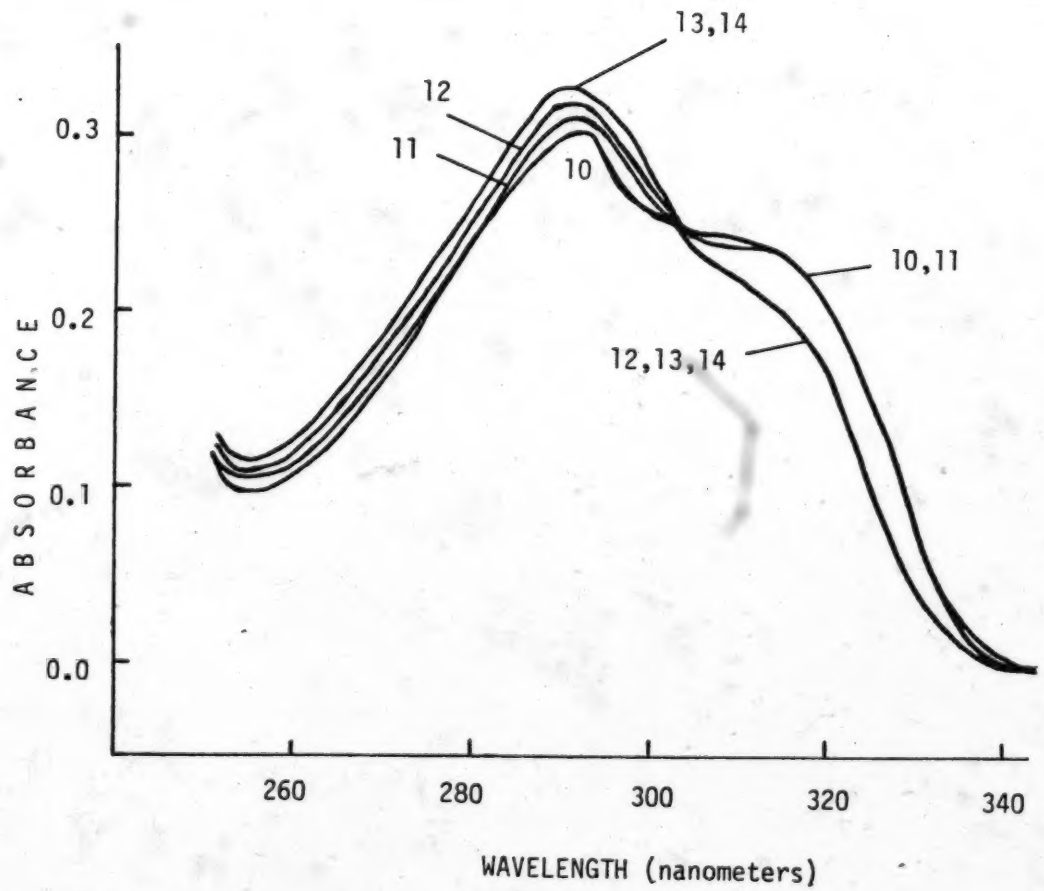
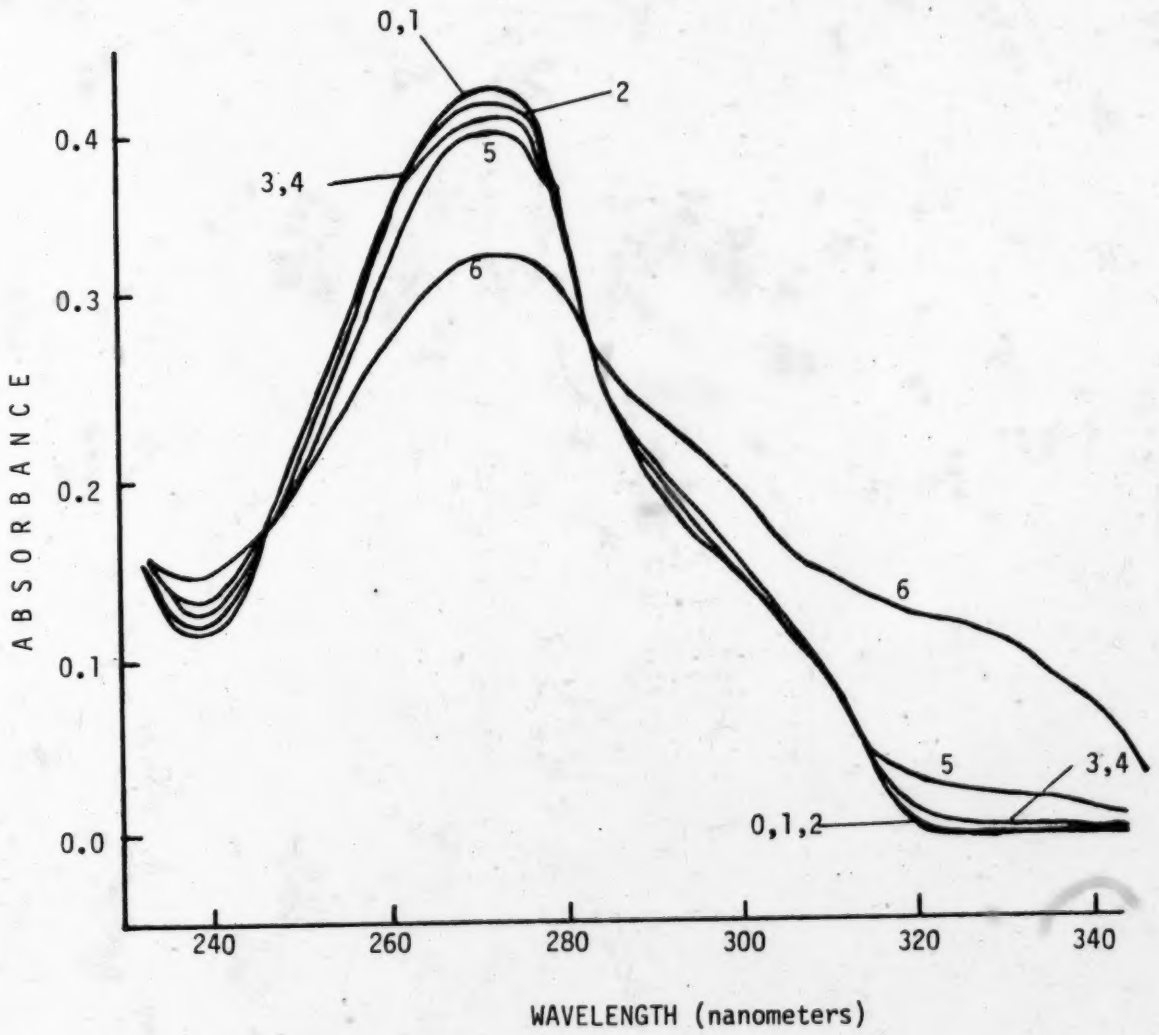


Figure 8. Absorption Spectra of Flumethiazide 2 x 10^{-5} M at Various pH Values



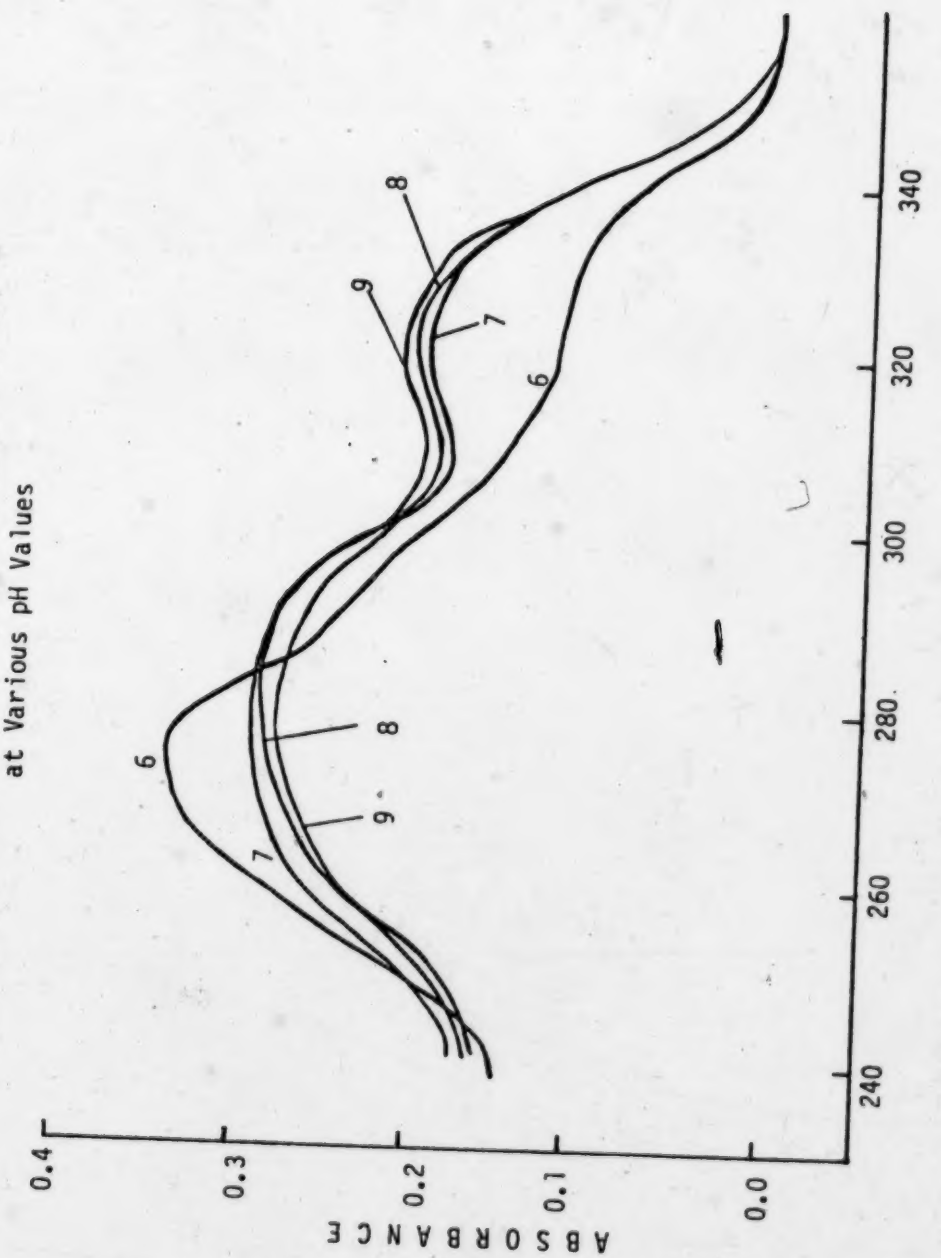


Figure 9. Absorption Spectra of Flumethiazide 2×10^{-5} M at Various pH Values

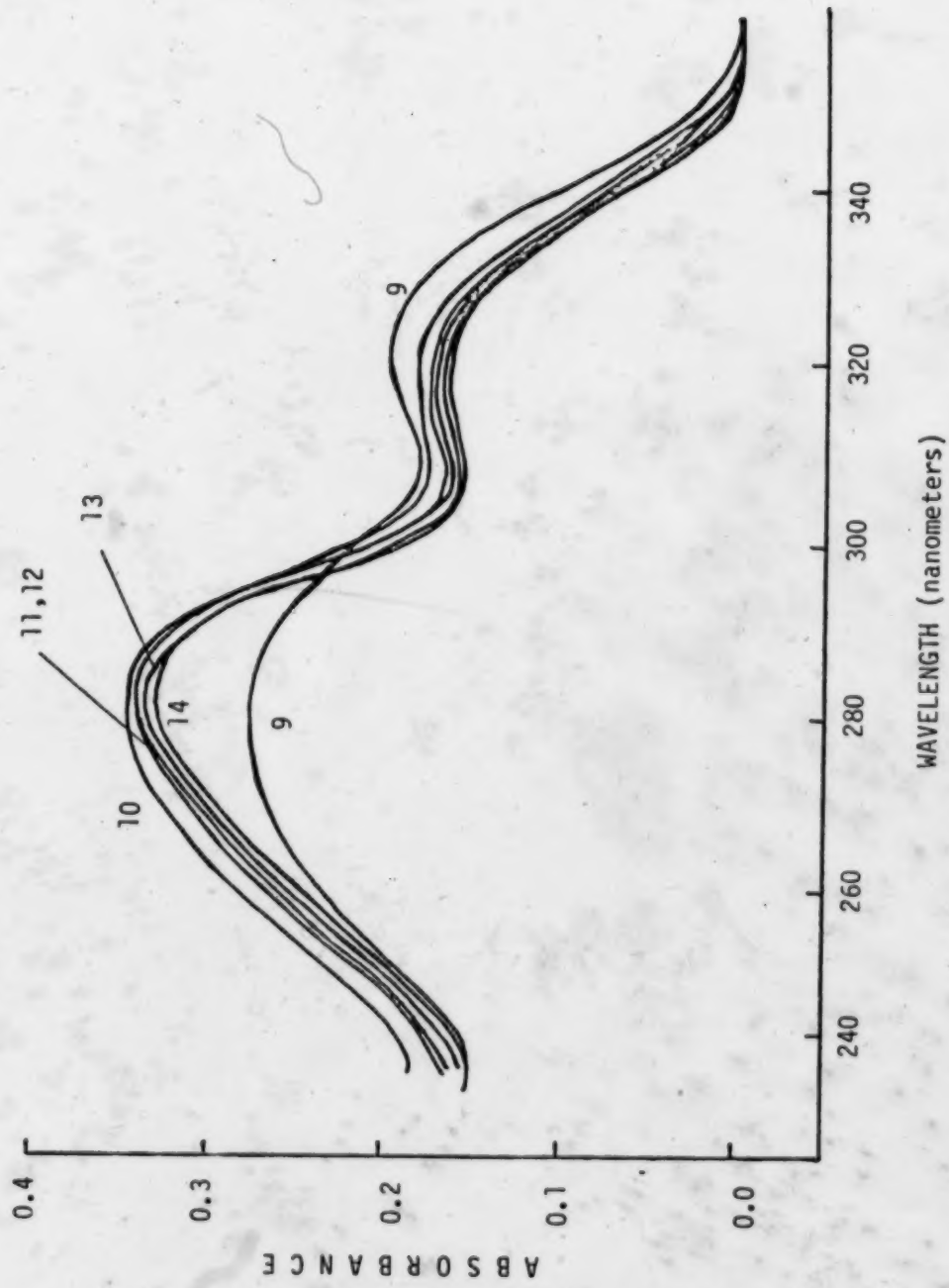


Figure 10. Absorption Spectra of Flumethiazide 2×10^{-5} M at Various pH Values

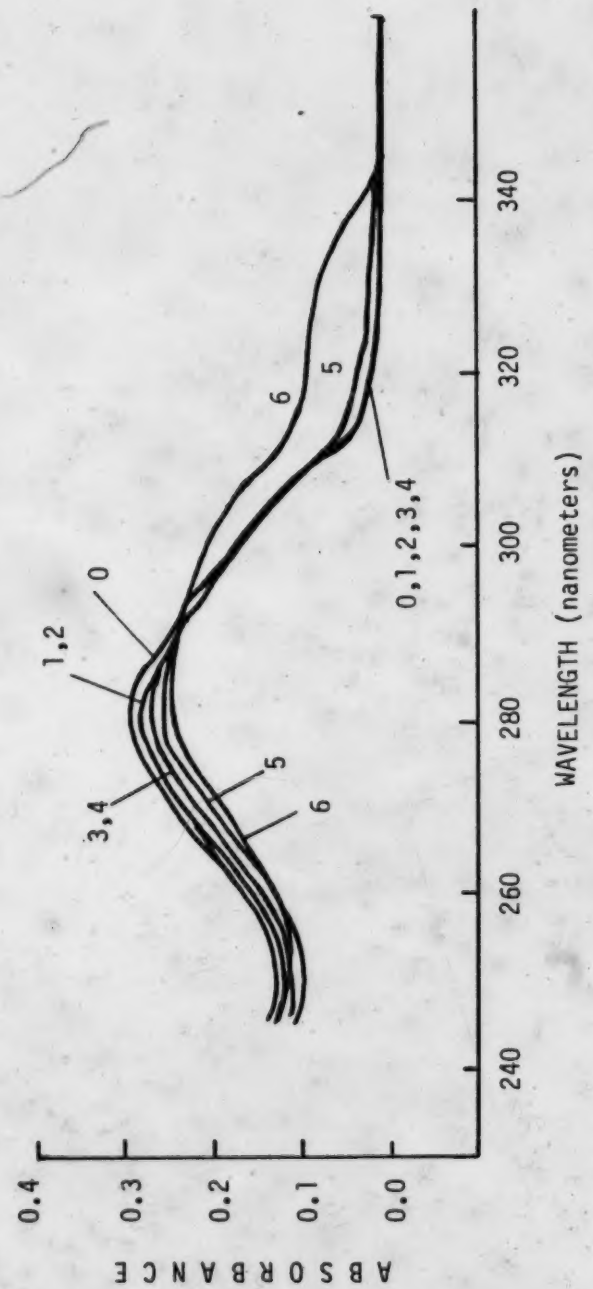


Figure 11. Absorption Spectra of Benzthiazide 2 x 10^{-5} M at Various pH Values

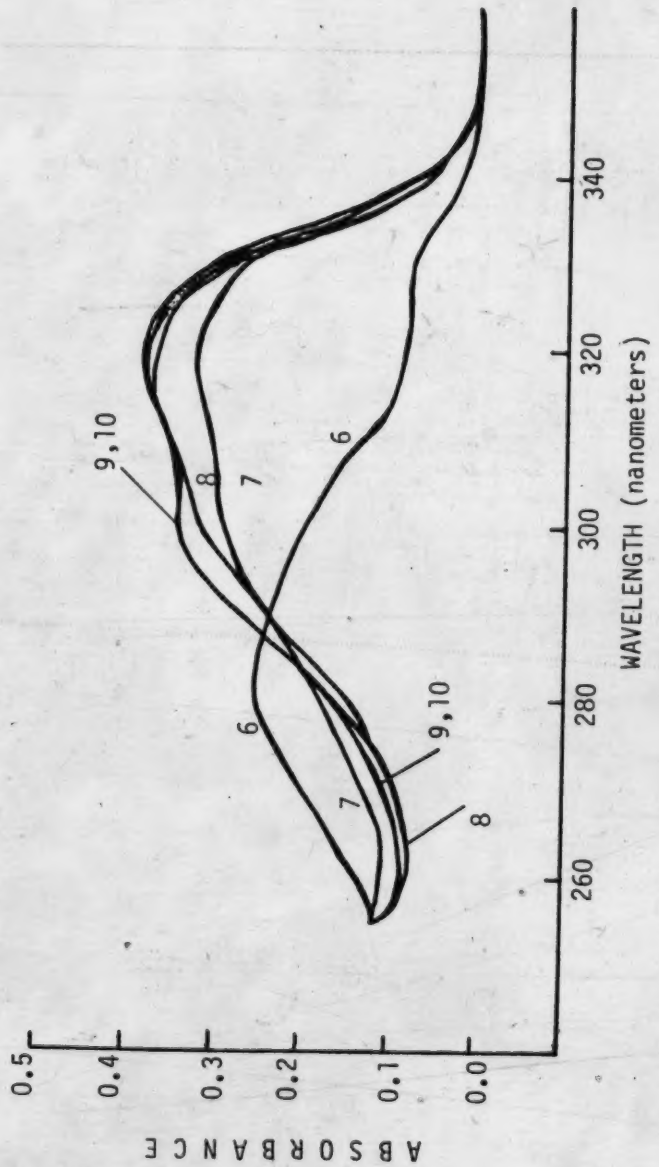


Figure 12. Absorption Spectra of Benzthiazide 2×10^{-5} M at Various pH Values

Figure 13. Absorption Spectra of Benzthiazide 2 x 10^{-5} M at Various pH Values

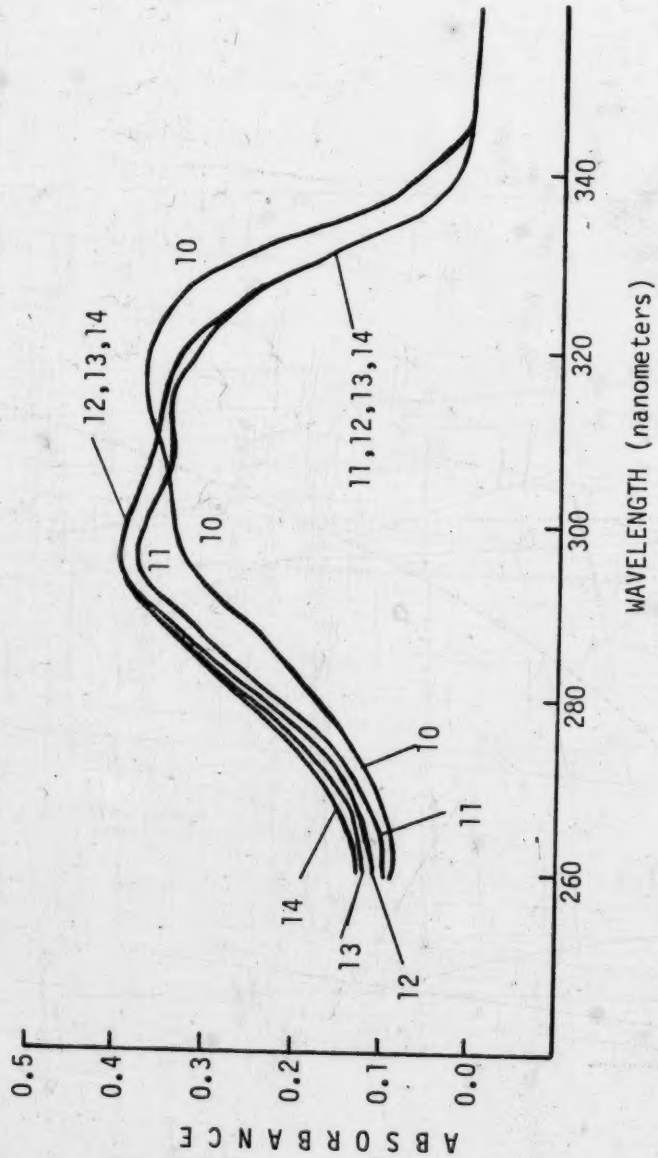


Figure 14. Absorption Spectra of Hydrochlorothiazide 2×10^{-5} M at Various pH Values

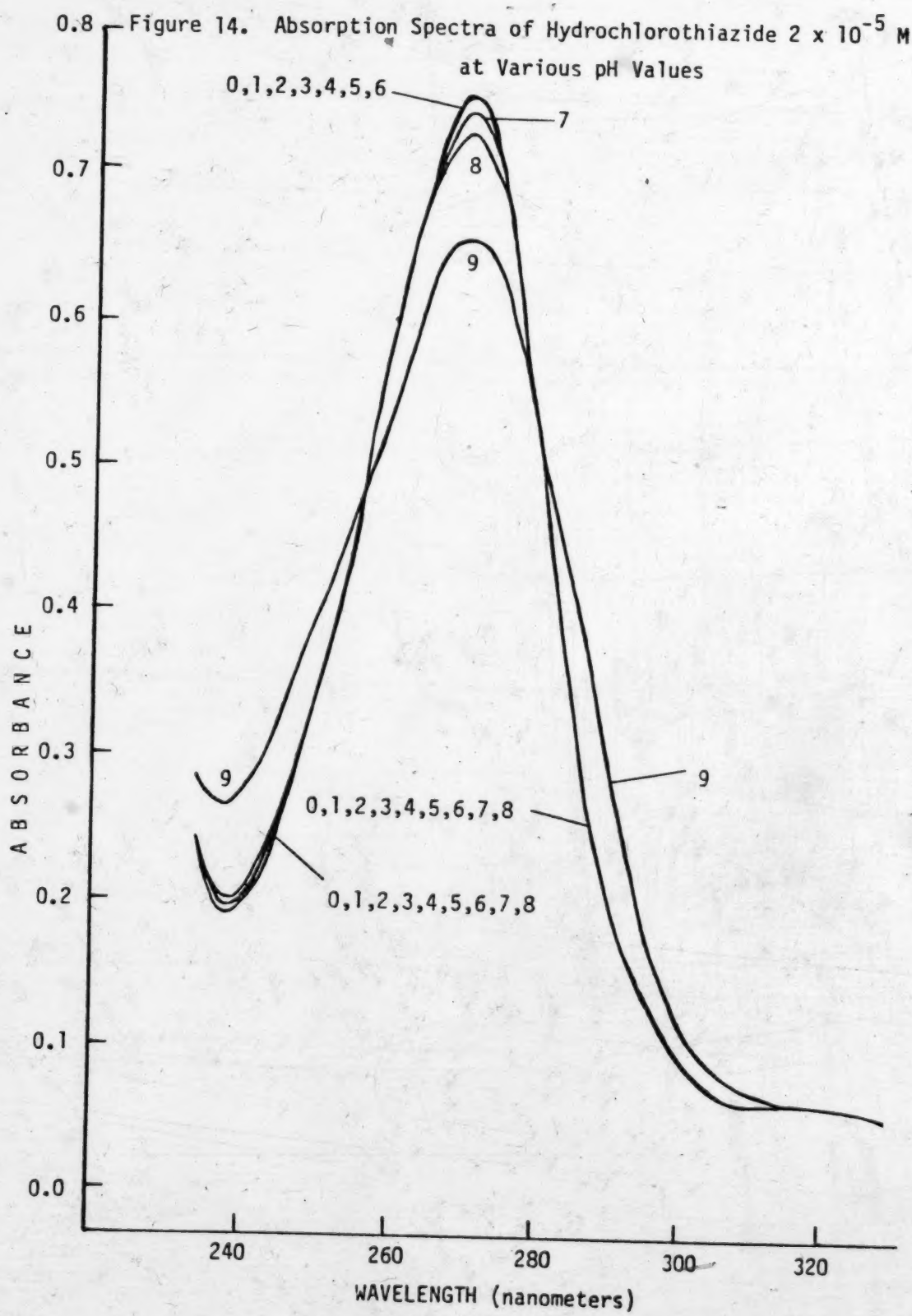


Figure 15. Absorption Spectra of Hydrochlorothiazide 2 x 10^{-5} M at Various pH Values

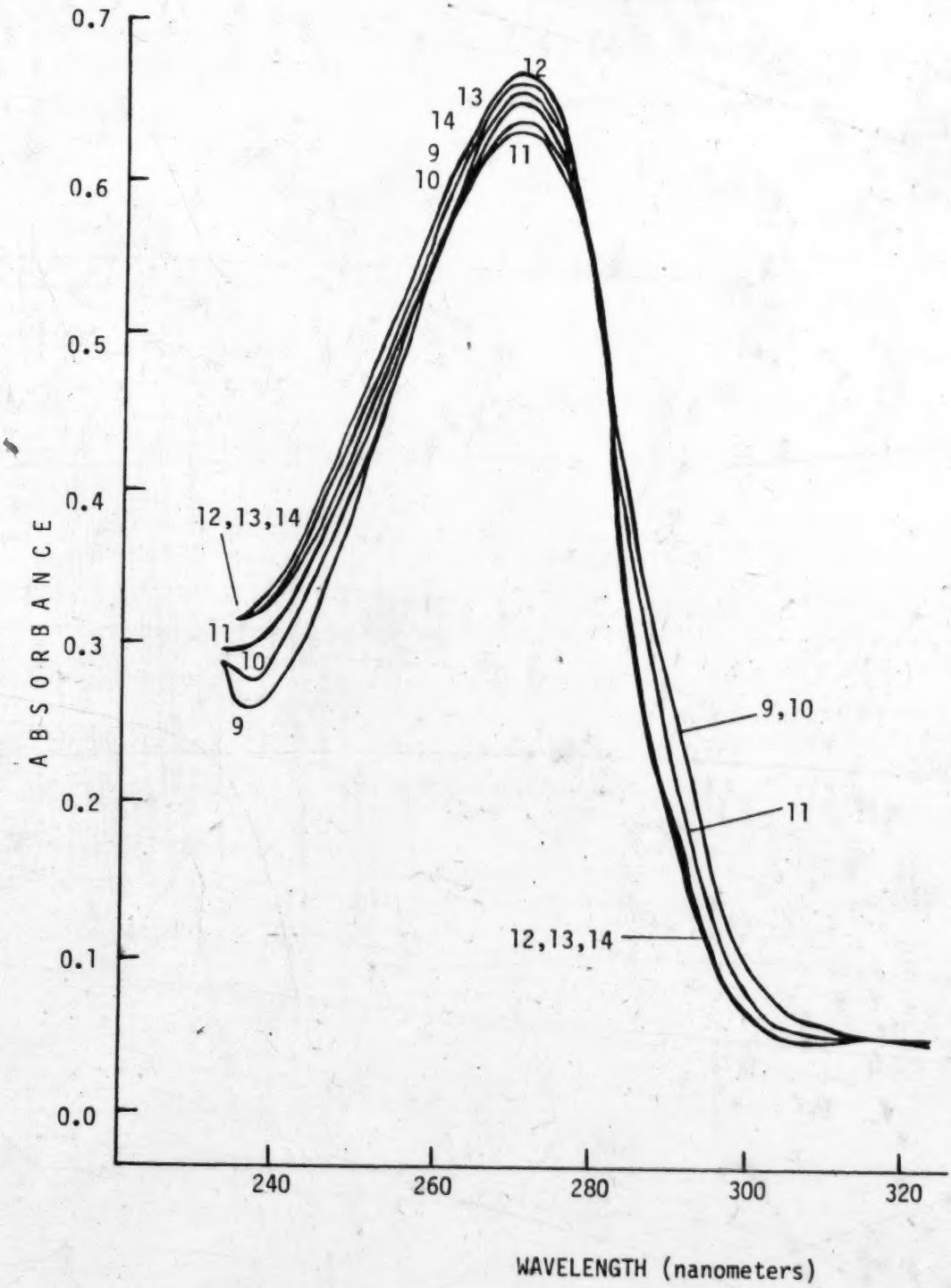


Figure 16. Absorption Spectra of Hydroflumethiazide 2×10^{-5} M at Various pH Values

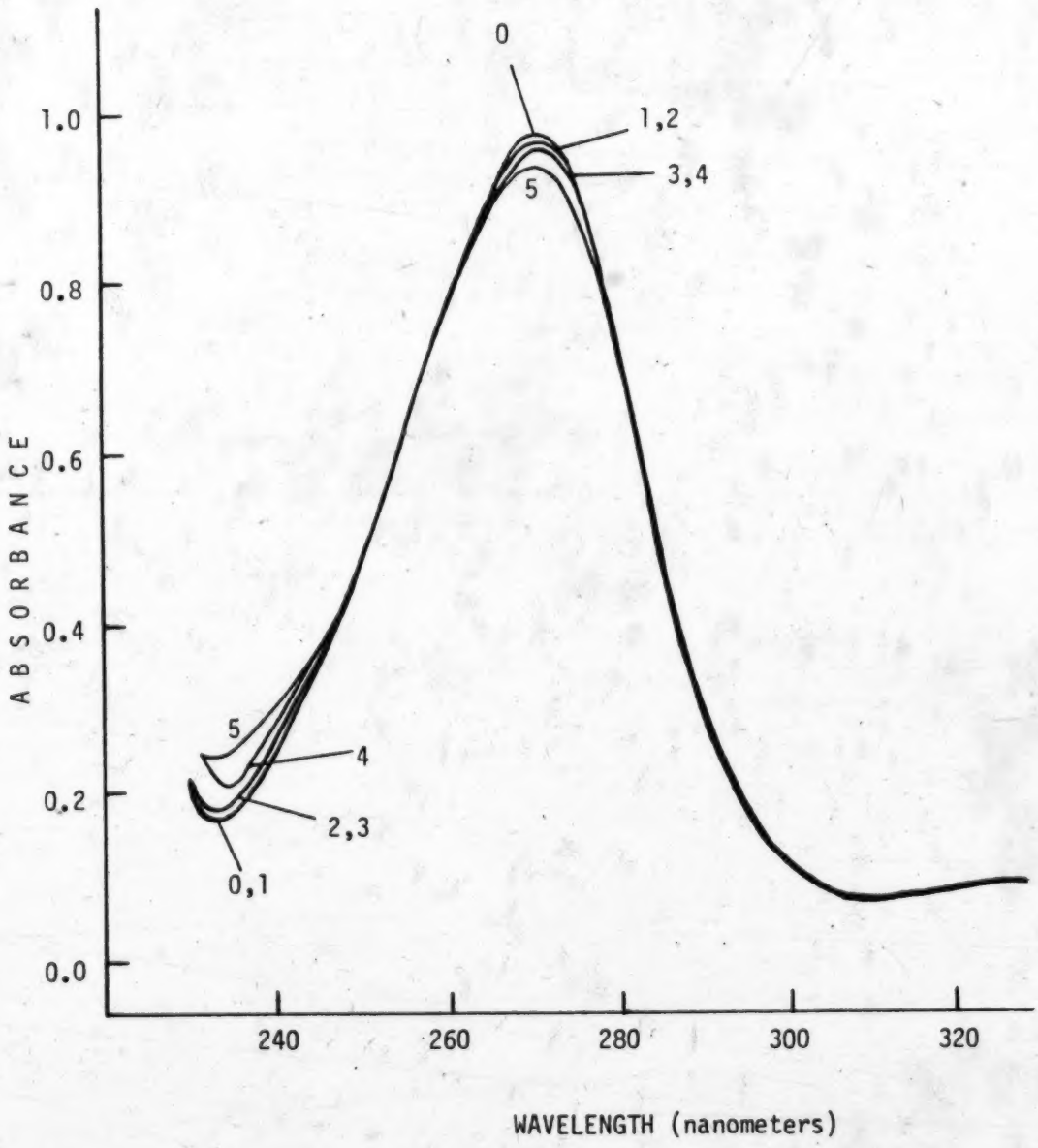


Figure 17. Absorption Spectra of Hydroflumethiazide 2×10^{-5} M at Various pH Values

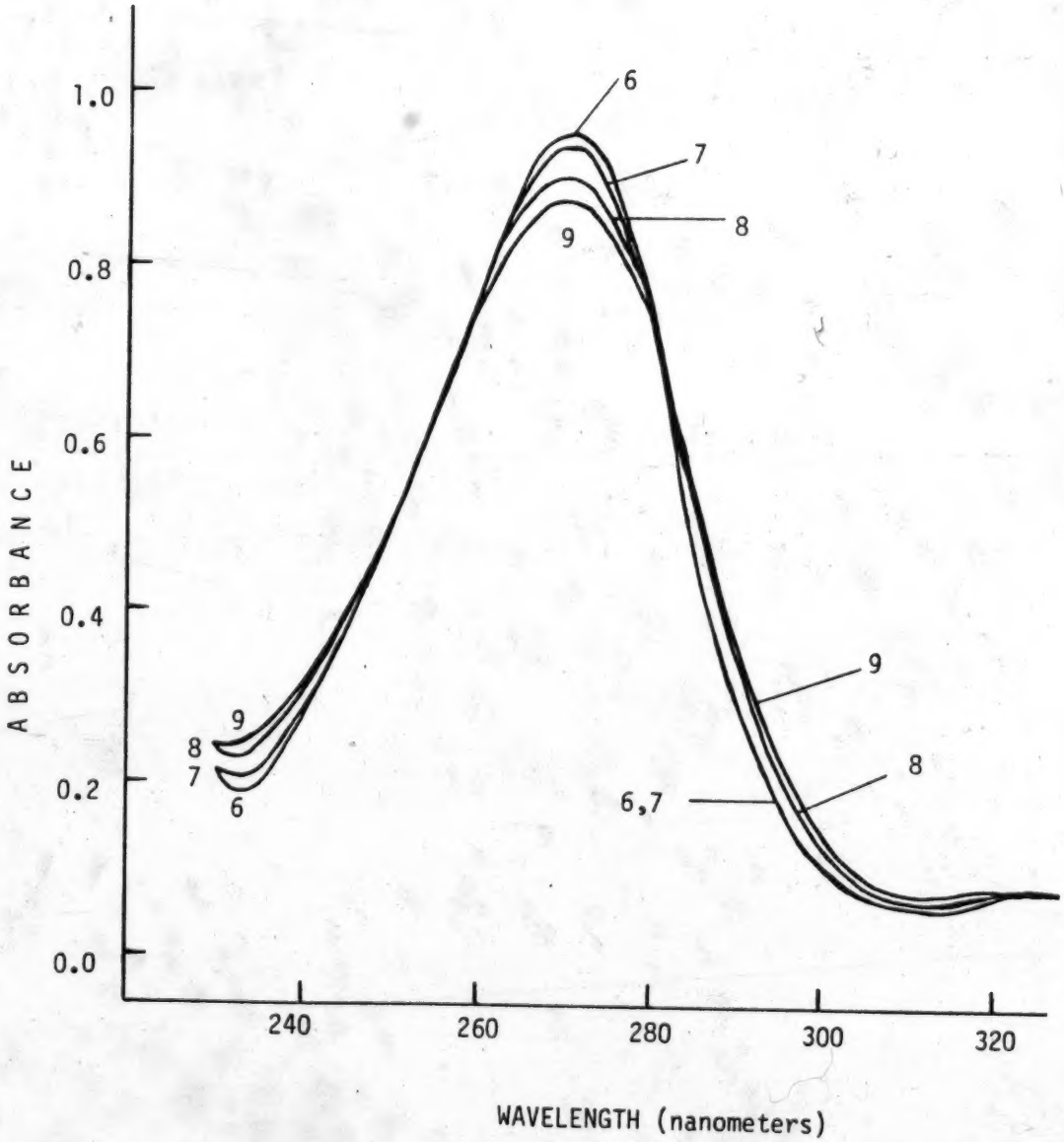


*Figure 18. Absorption Spectra of Hydroflumethiazide 2 x 10^{-5} M at Various pH Values

* Obtained under different experimental conditions than the spectra in Figures 16 and 17.

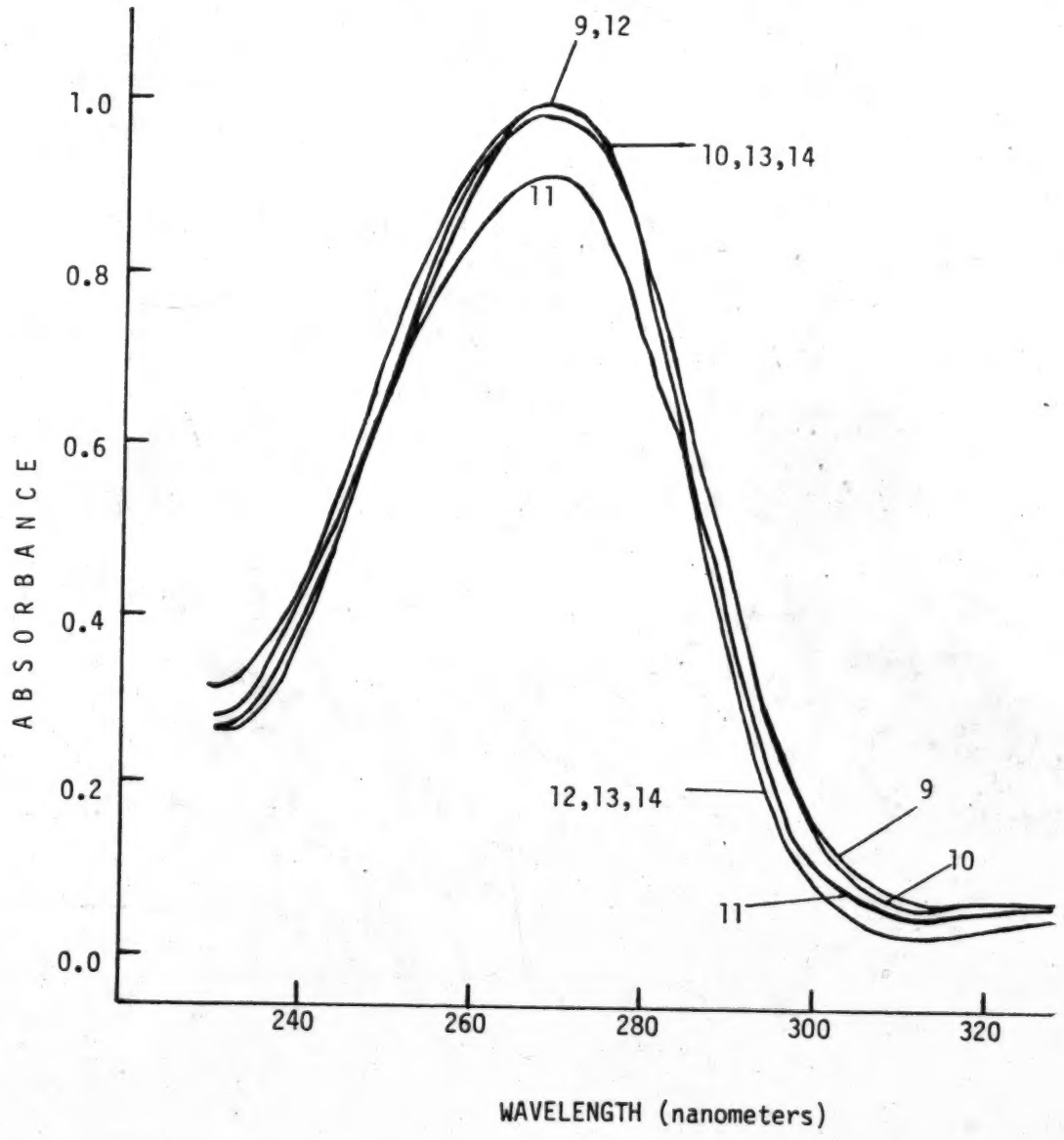


Figure 19. Absorption Spectra of Bendroflumethiazide 2 x 10^{-5} M at Various pH Values

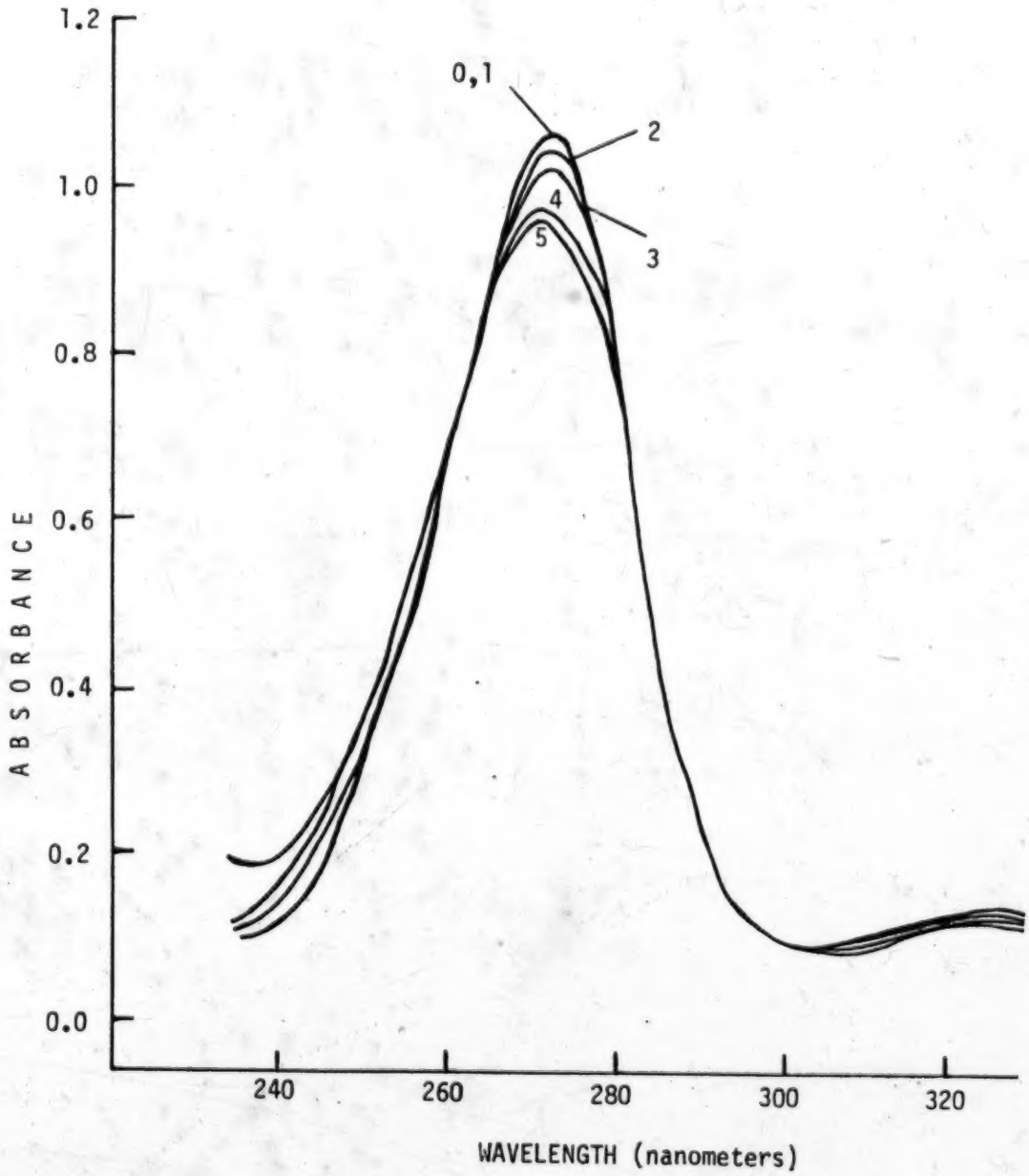
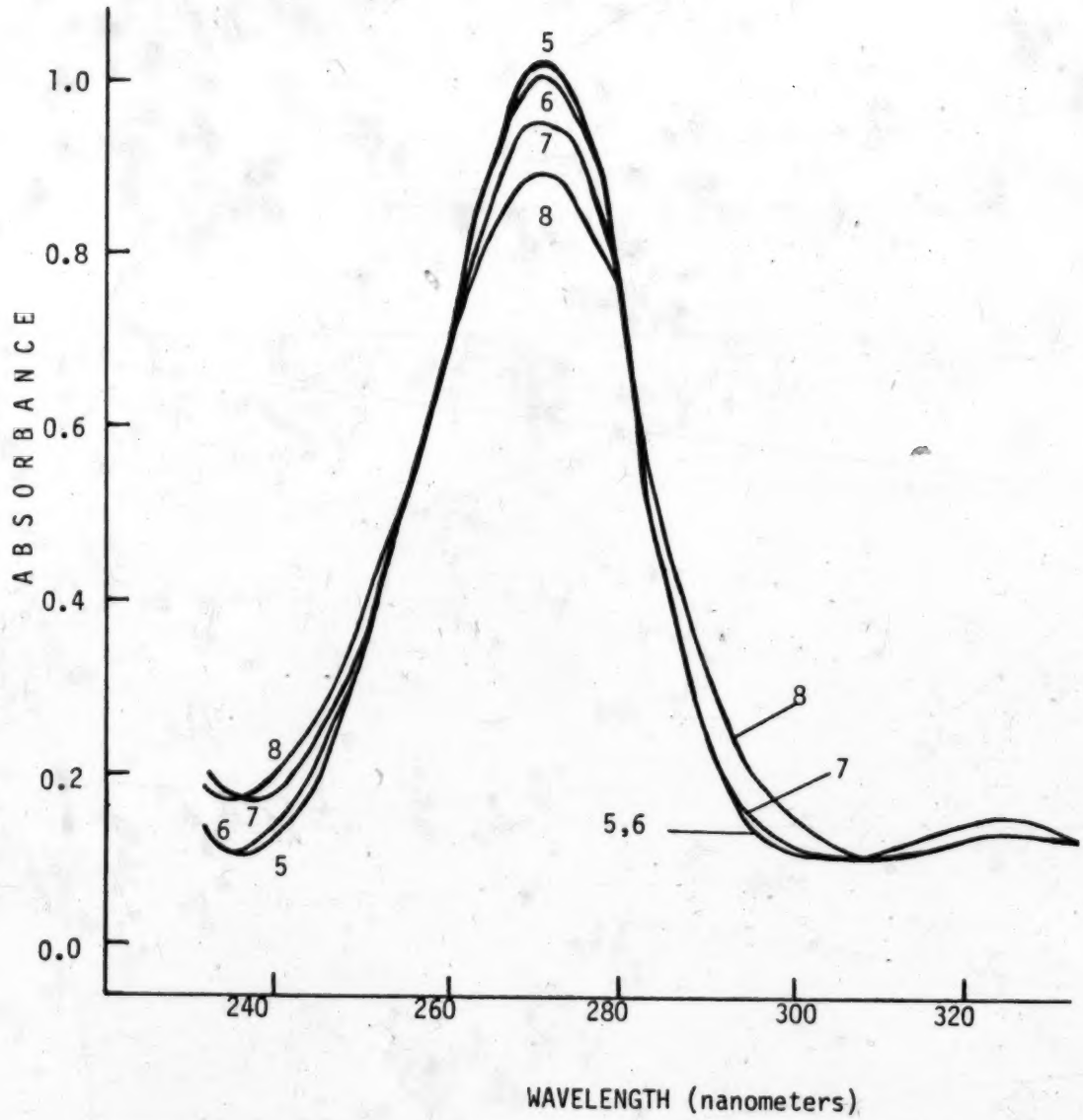


Figure 20. Absorption Spectra of Bendroflumethiazide 2 x 10^{-5} M at Various pH Values



* Figure 21. Absorption Spectra of Bendroflumethiazide 2×10^{-5} M at Various pH Values

* Obtained at a different recorder calibration than the spectra in Figures 19 and 20.

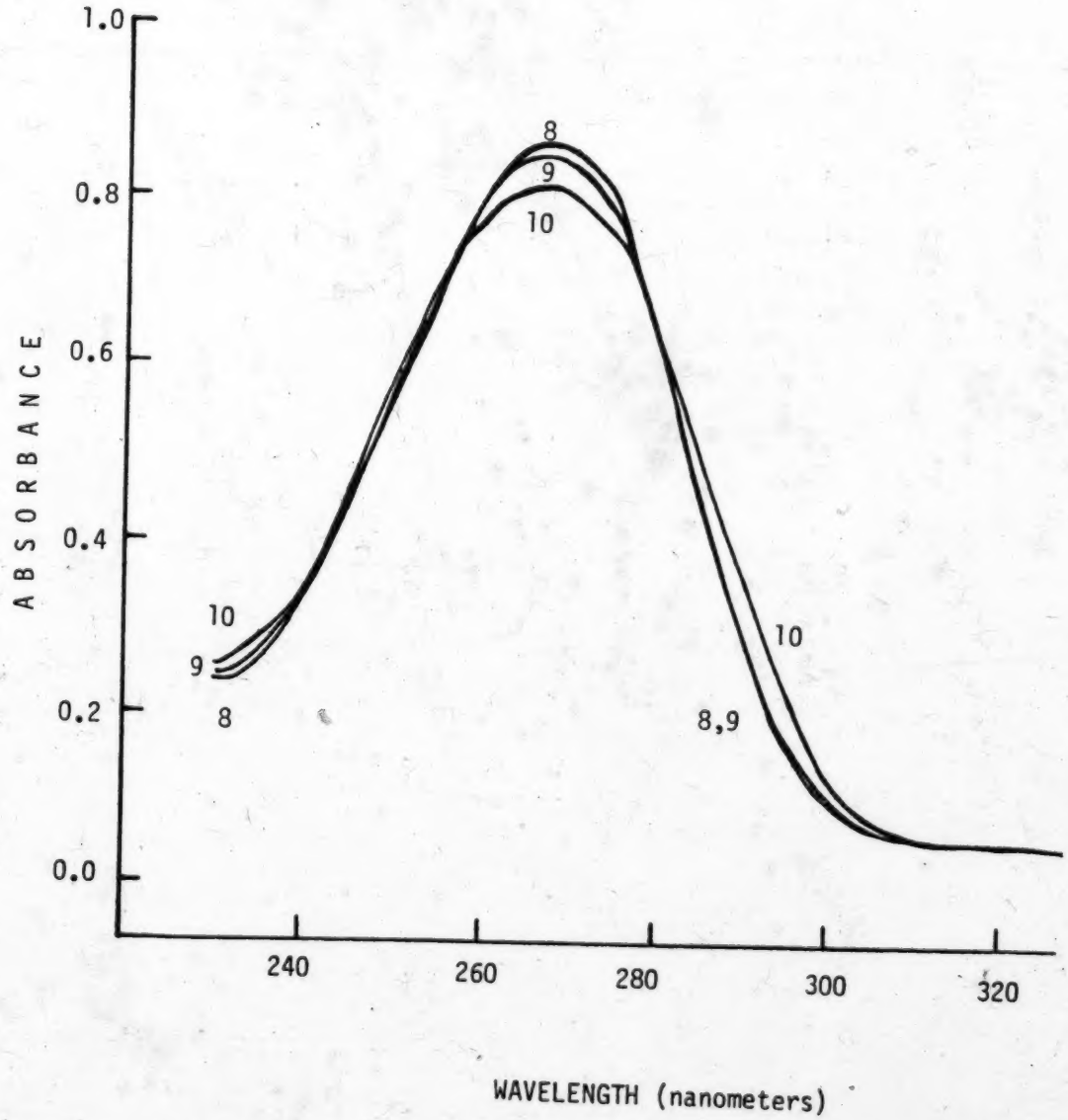


*Figure 22. Absorption Spectra of Bendroflumethiazide 2 x 10^{-5} M at Various pH Values

* Obtained under the same conditions as the spectra in Figure 21.

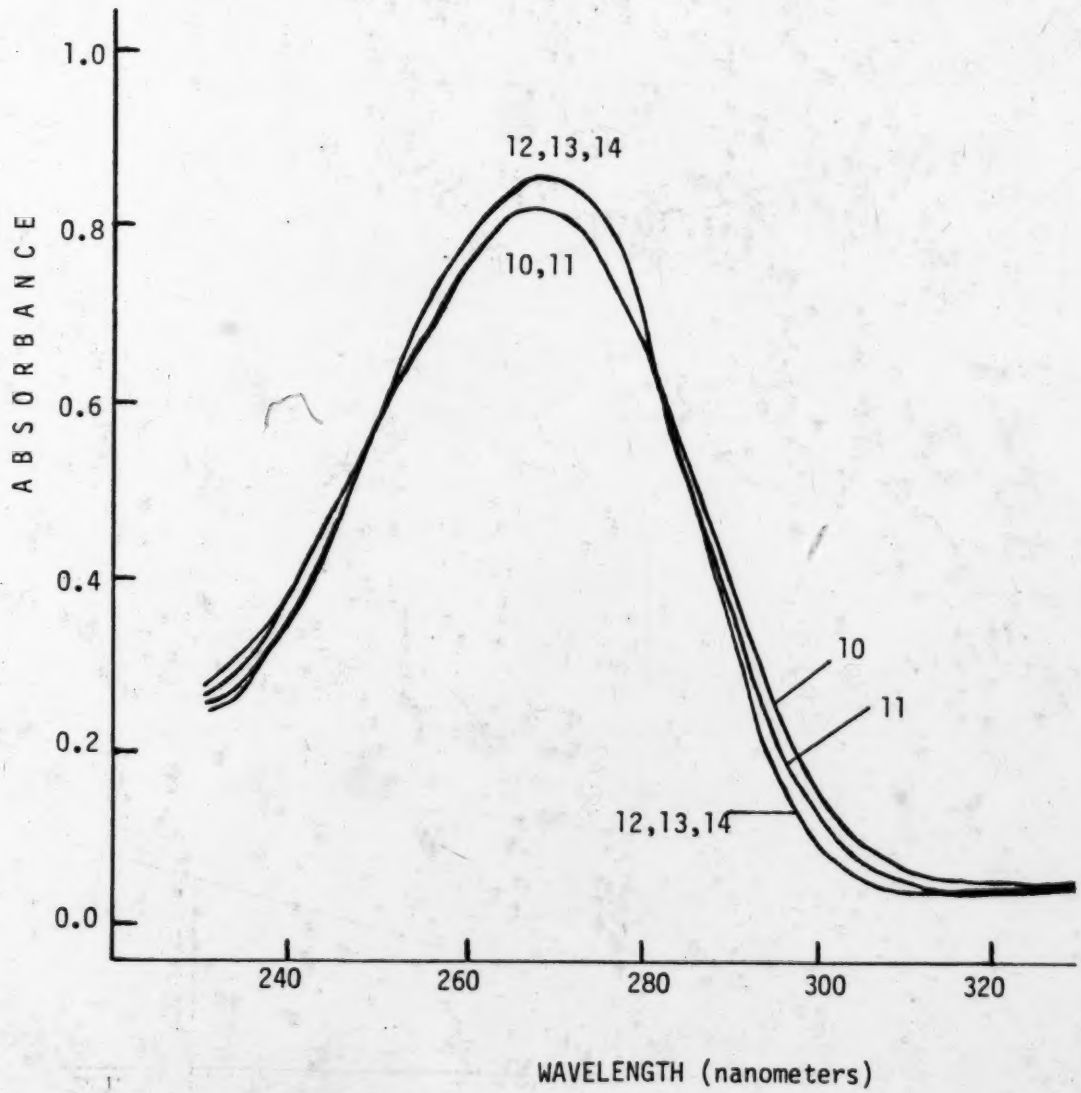


Figure 23. Absorption Spectra of Cyclothiazide 2×10^{-5} M at Various pH Values

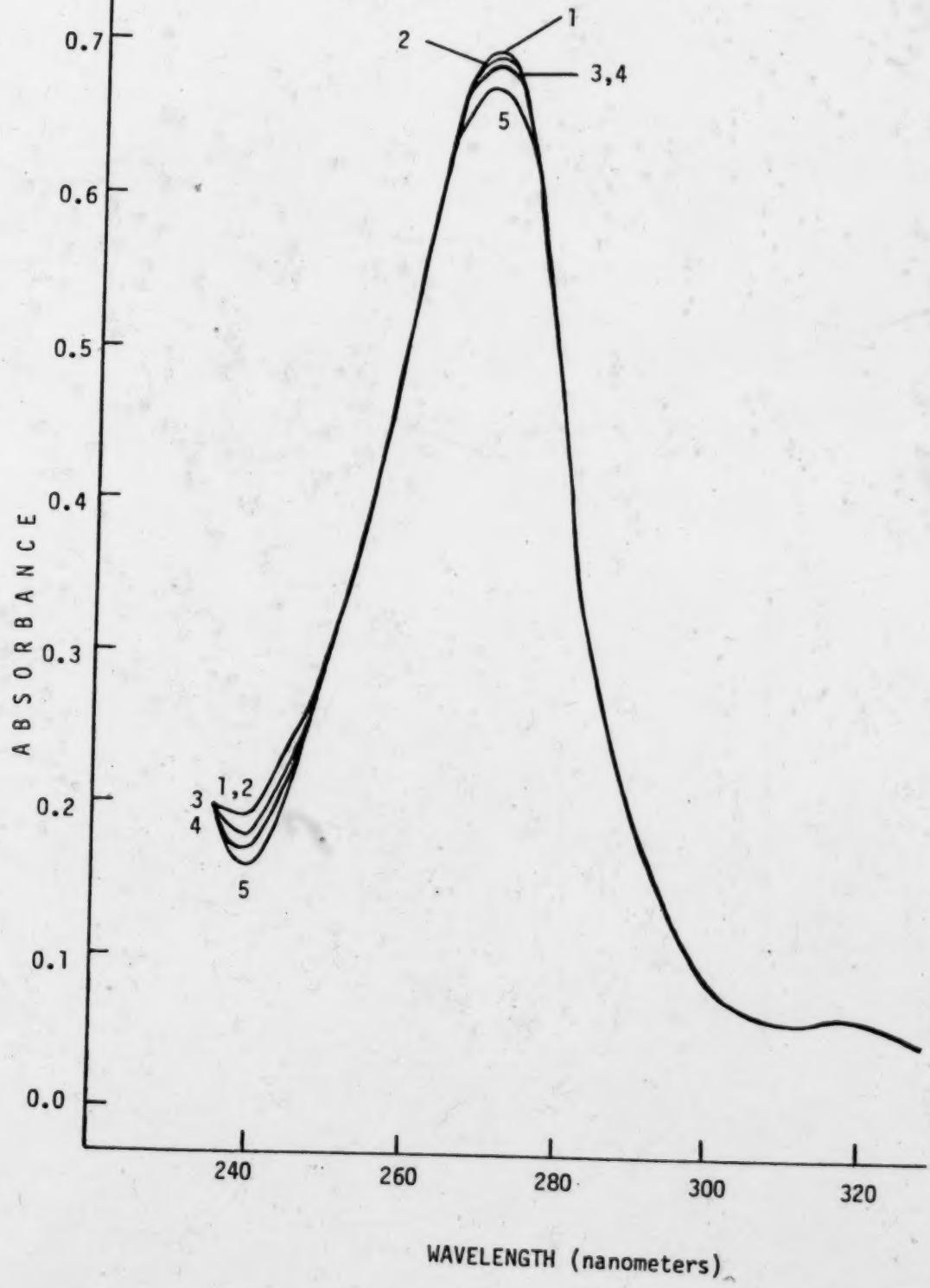


Figure 24. Absorption Spectra of Cyclothiazide 2 x 10^{-5} M at Various pH Values

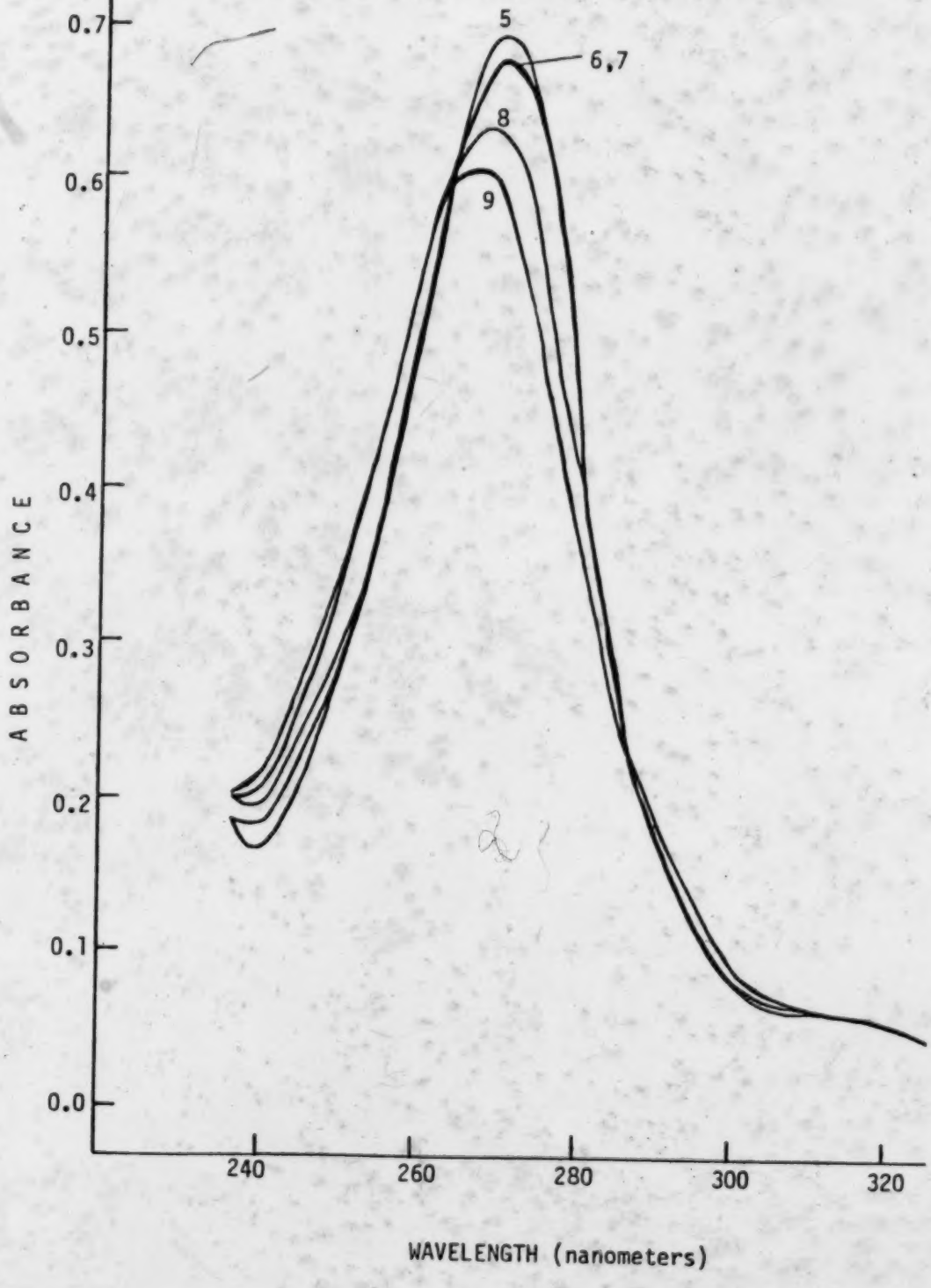


Figure 25. Absorption Spectra of Cyclothiazide 2 x 10^{-5} M at Various pH Values

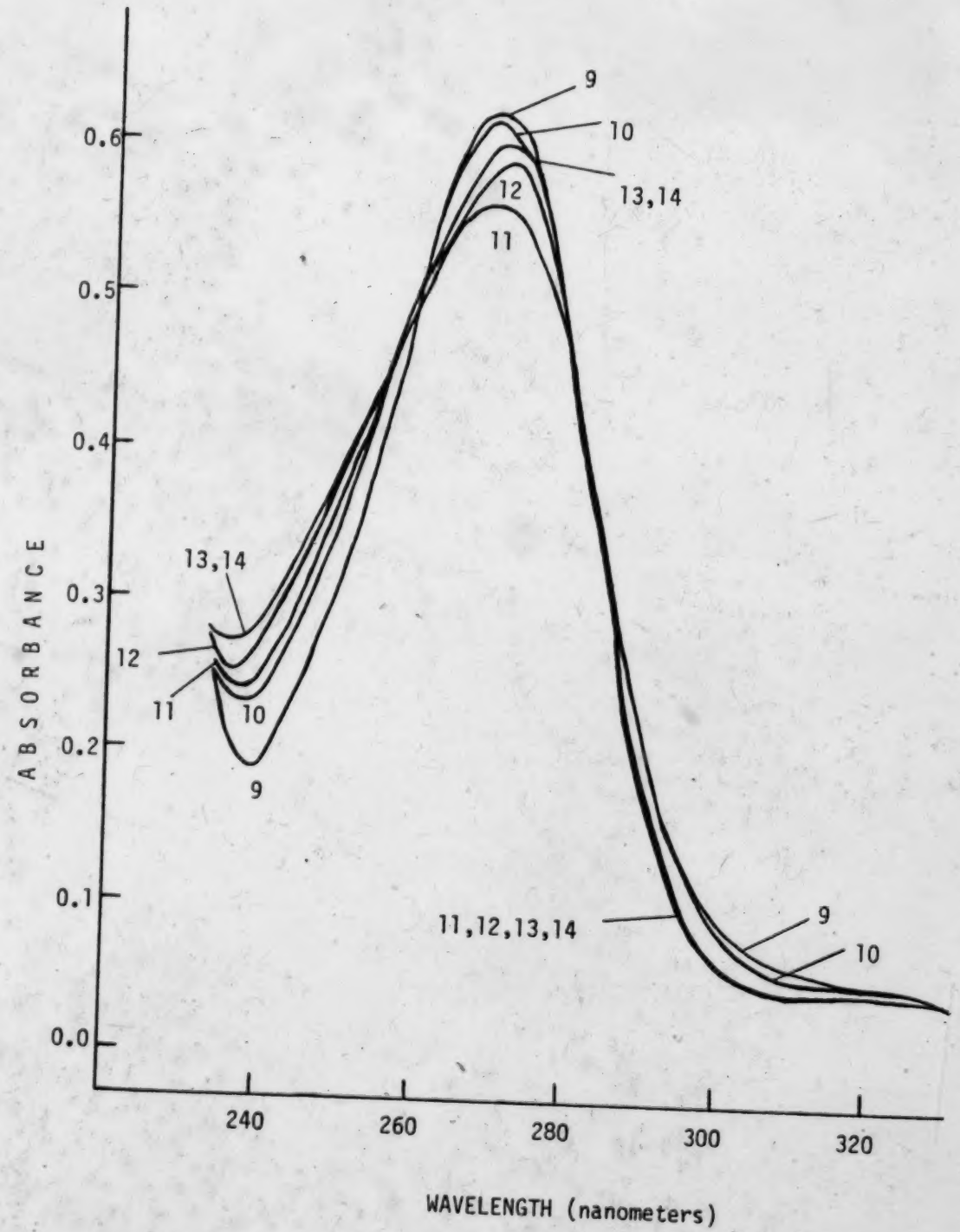


Figure 26. Absorption Spectra of Cyclopenthiazide 2 x 10^{-5} M at Various pH Values

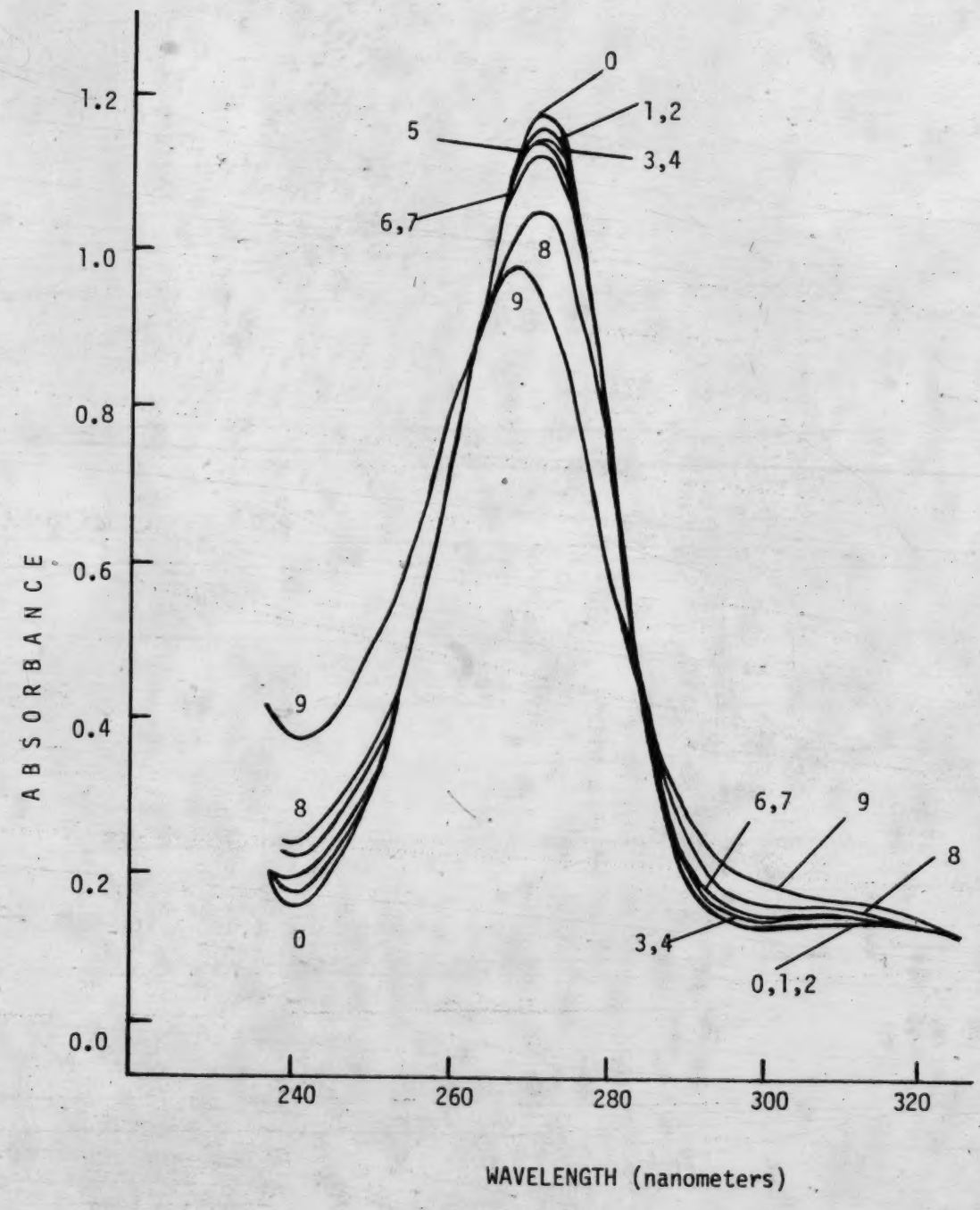


Figure 27. Absorption Spectra of Cyclopenthiazide 2 x 10^{-5} M at Various pH Values

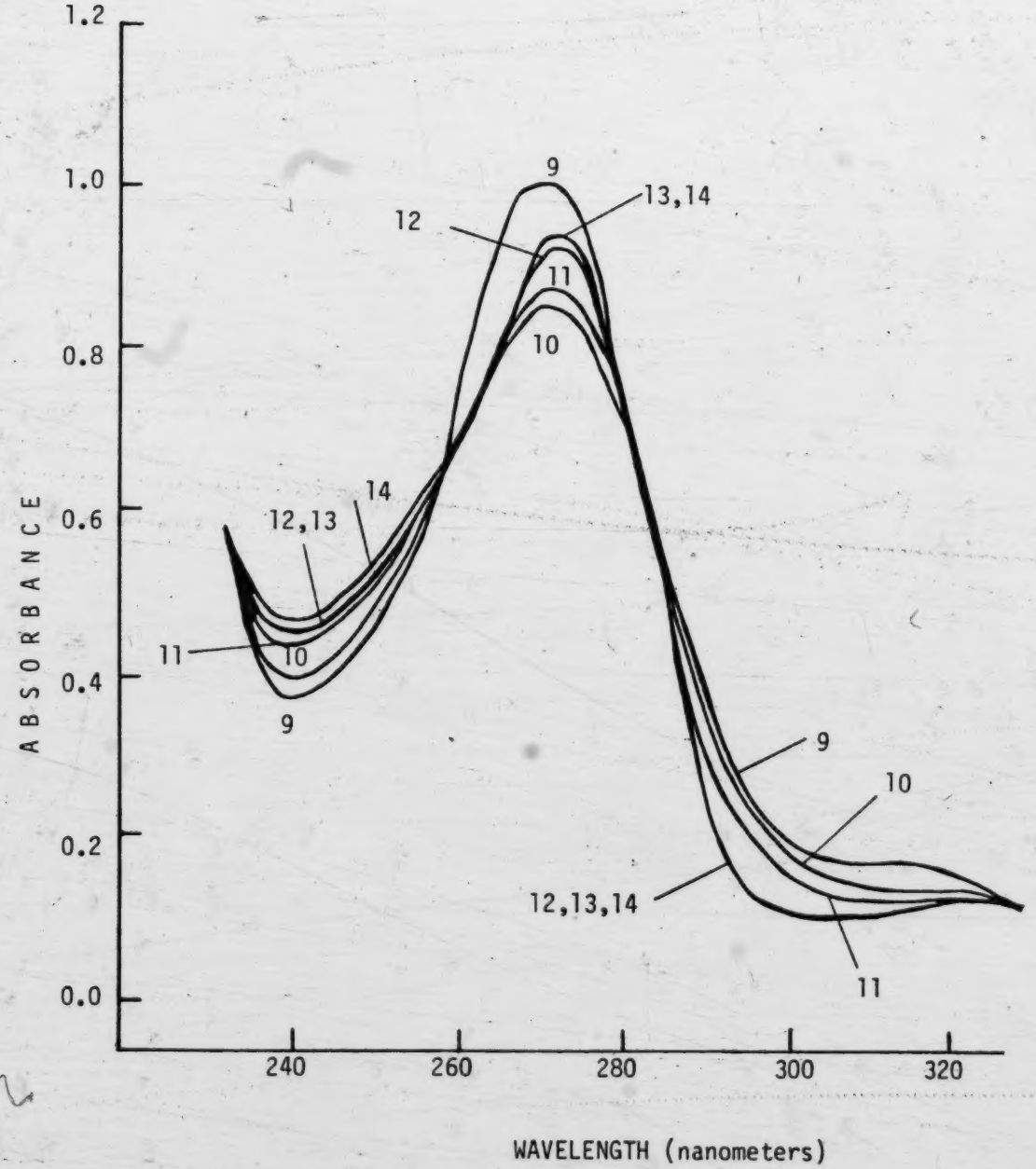


Figure 28. Absorption Spectra of Althiazide 2 x 10^{-5} M at Various pH Values

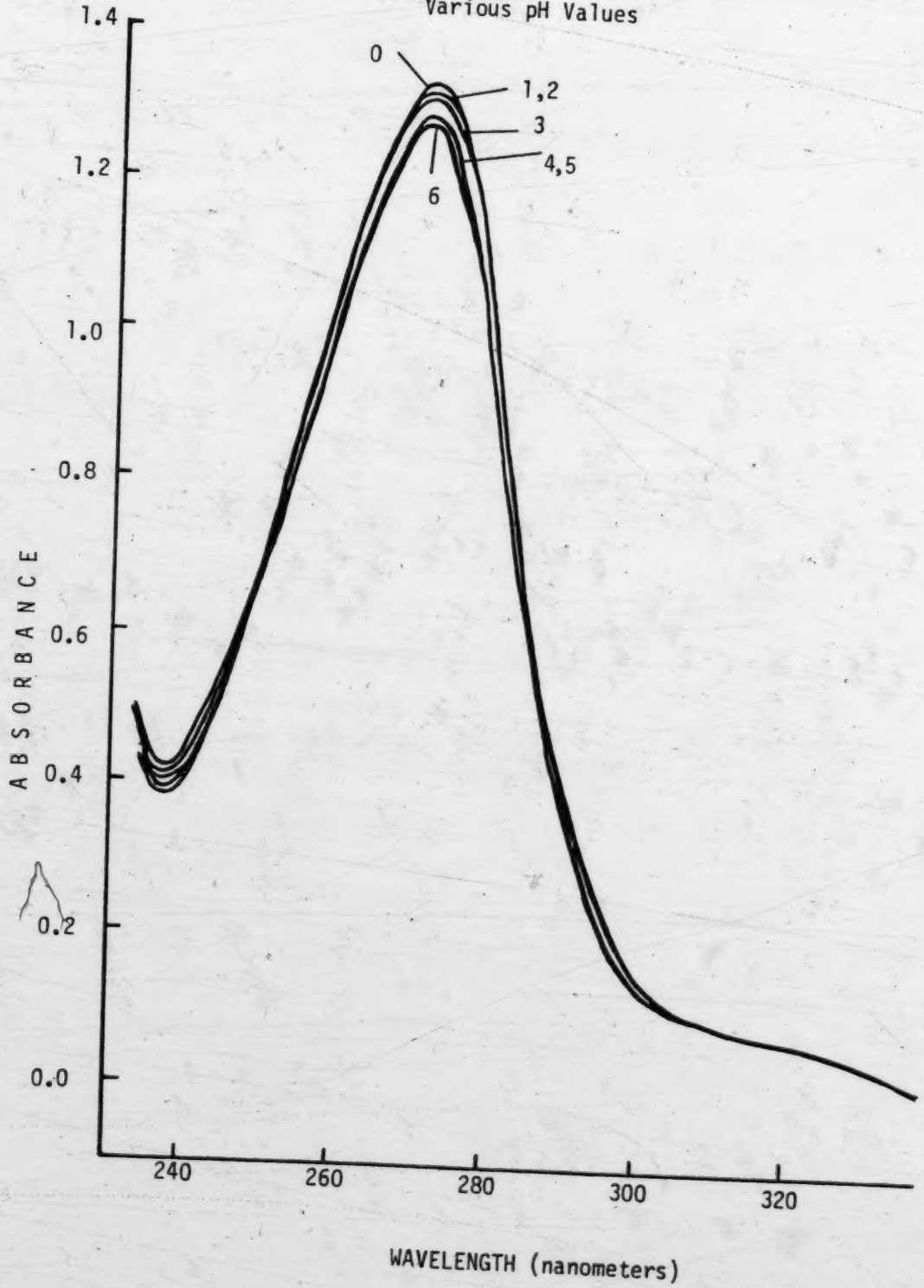


Figure 29. Absorption Spectra of Althiazide 2×10^{-5} M at Various pH Values

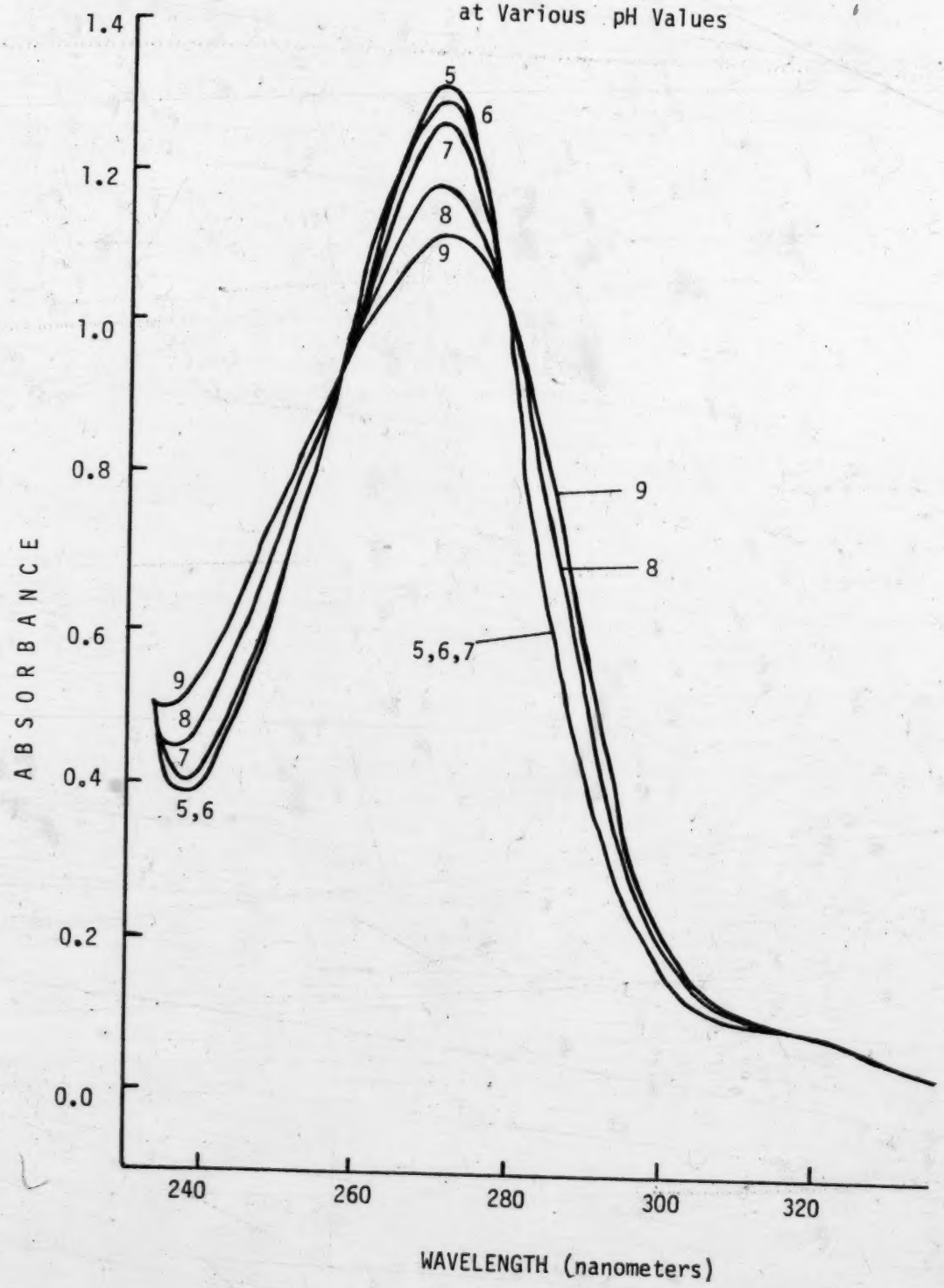


Figure 30. Absorption Spectra of Althiazide 2×10^{-5} M at Various pH Values

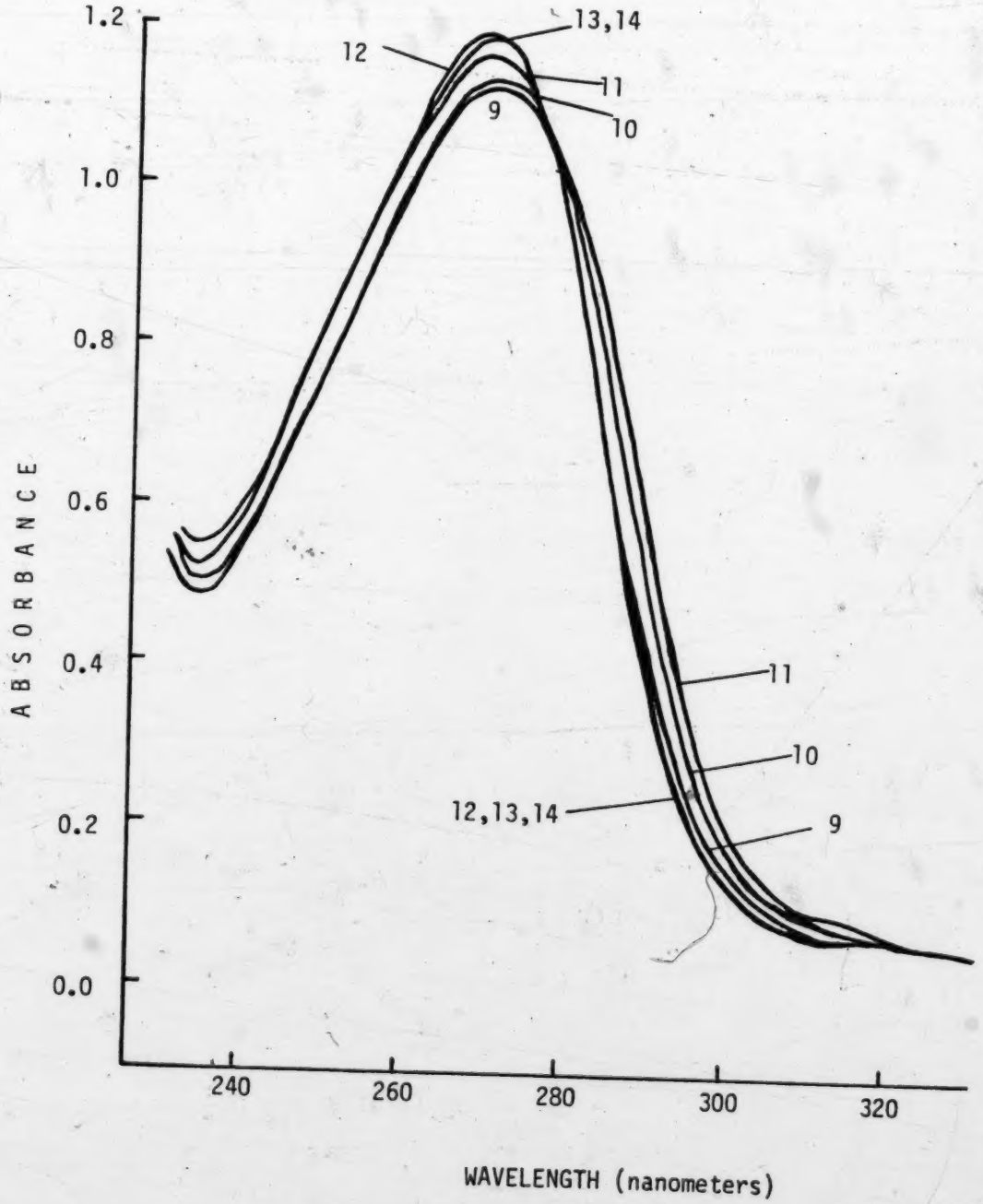


Figure 31. Absorption Spectra of Trichloromethiazide 2×10^{-5} M at Various pH Values

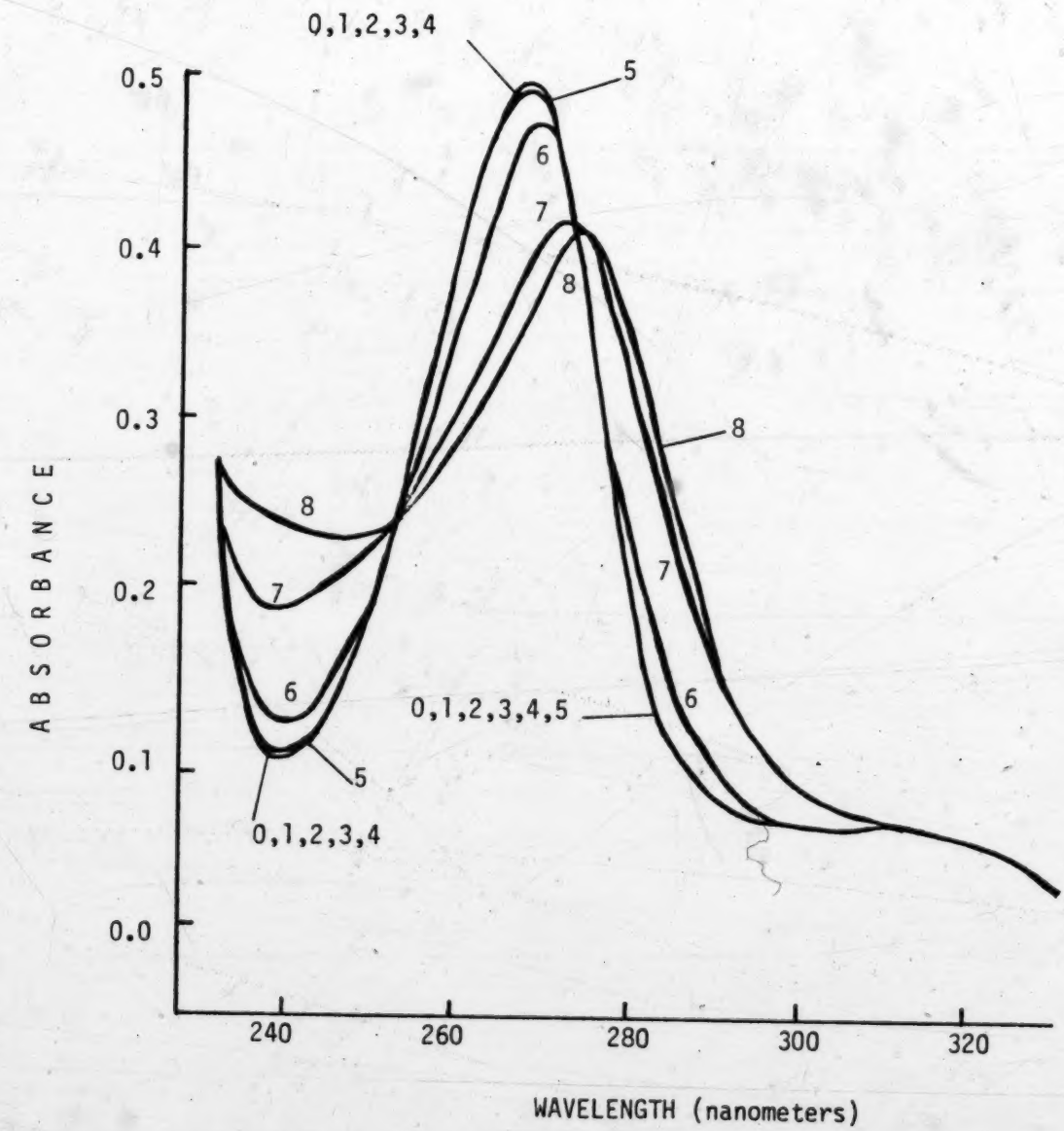


Figure 32. Absorption Spectra of Trichloromethiazide 2 x 10^{-5} M at Various pH Values

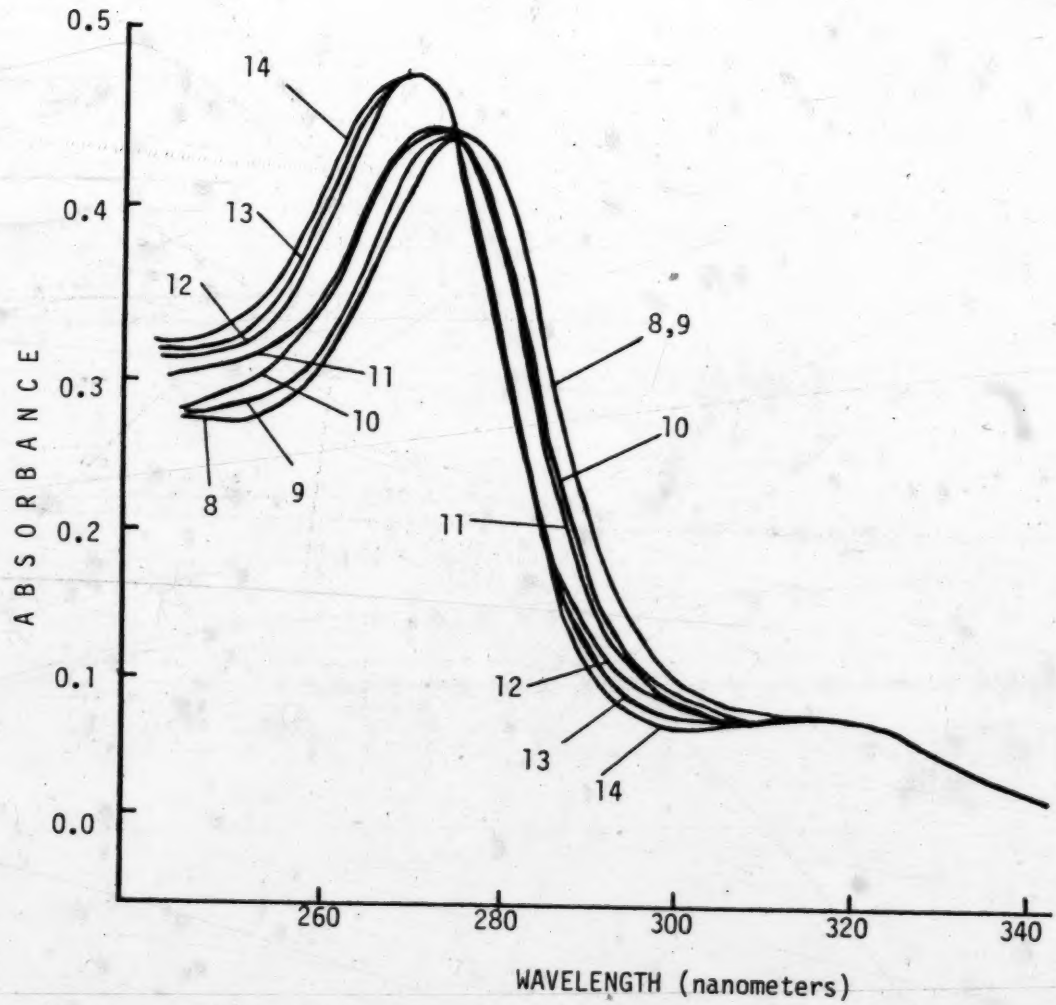


Figure 33. Absorption Spectra of Methyclothiazide 2×10^{-5} M at Various pH Values

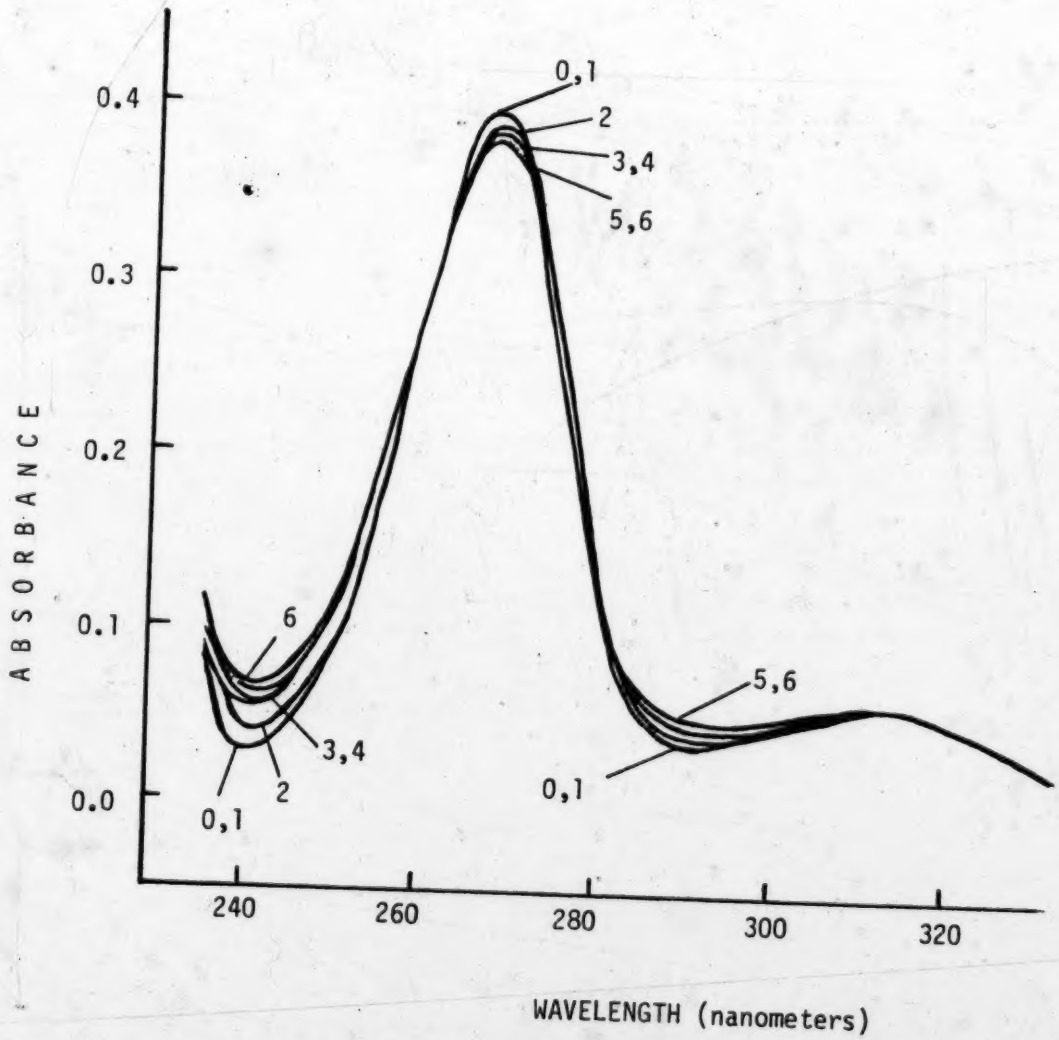


Figure 34. Absorption Spectra of Methyclothiazide 2 x 10^{-5} M at Various pH Values

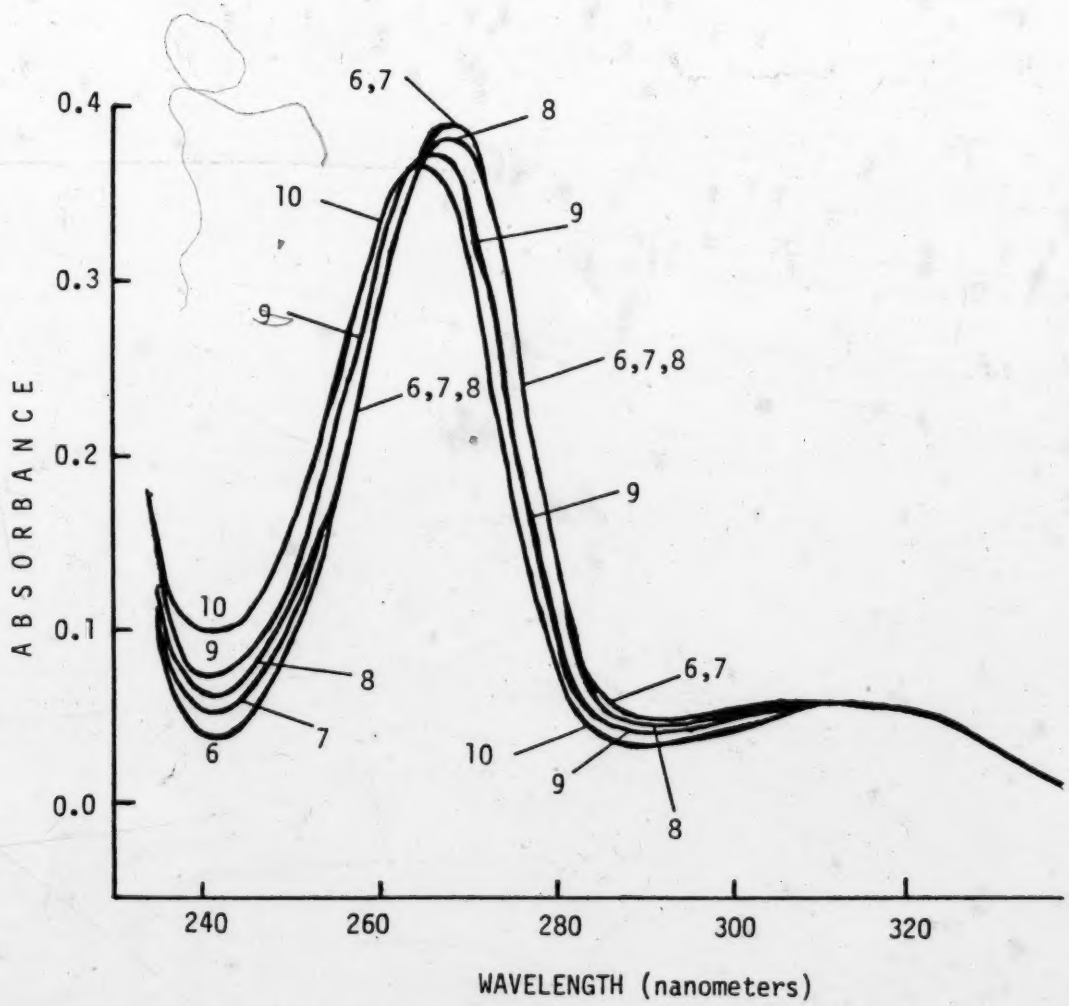
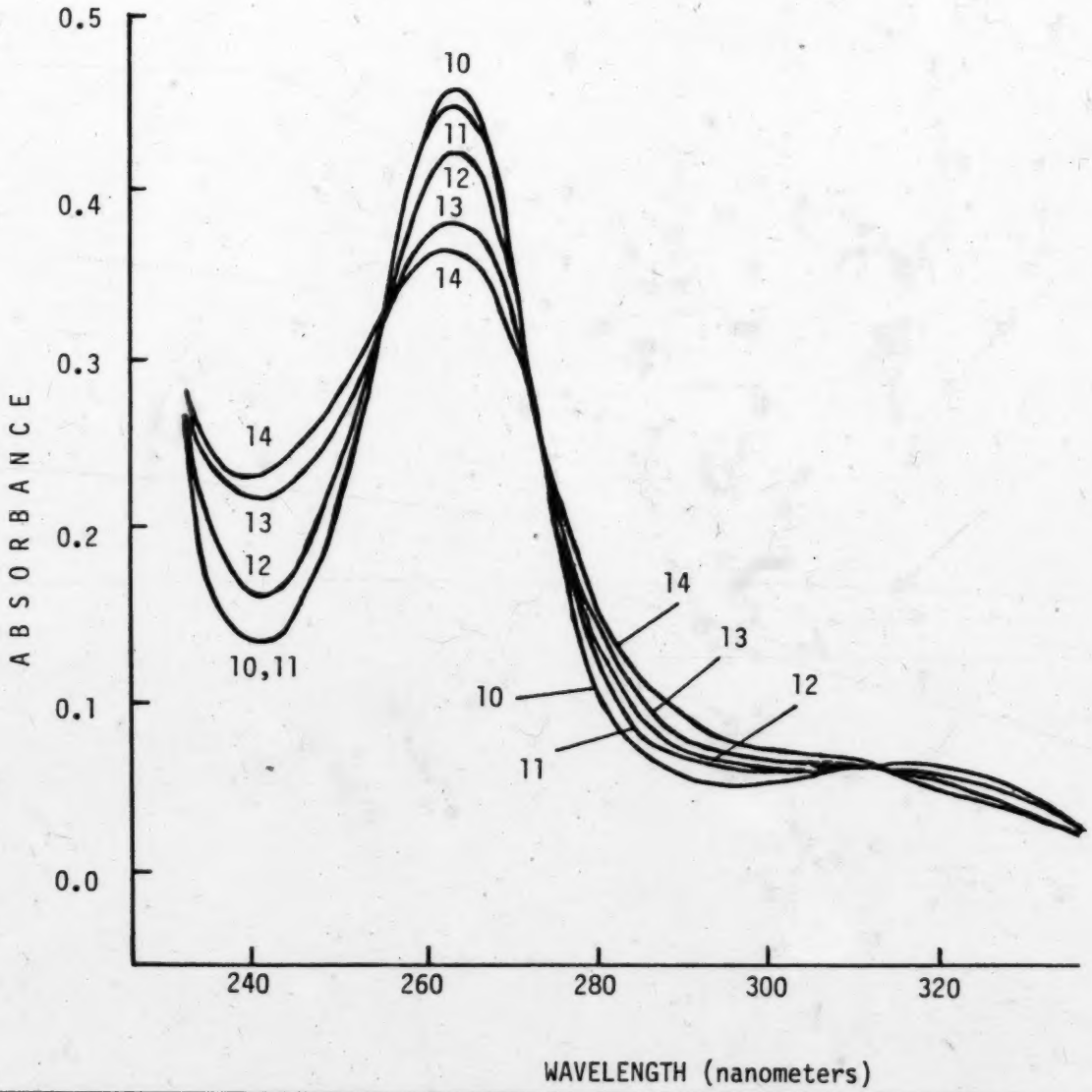


*Figure 35. Absorption Spectra of Methyclothiazide 2 x 10^{-5} M at Various pH Values



* Obtained under different experimental conditions than the spectra in Figures 33 and 34.

Figure 36. Absorption Spectra of Polythiazide 2×10^{-5} M at Various pH Values

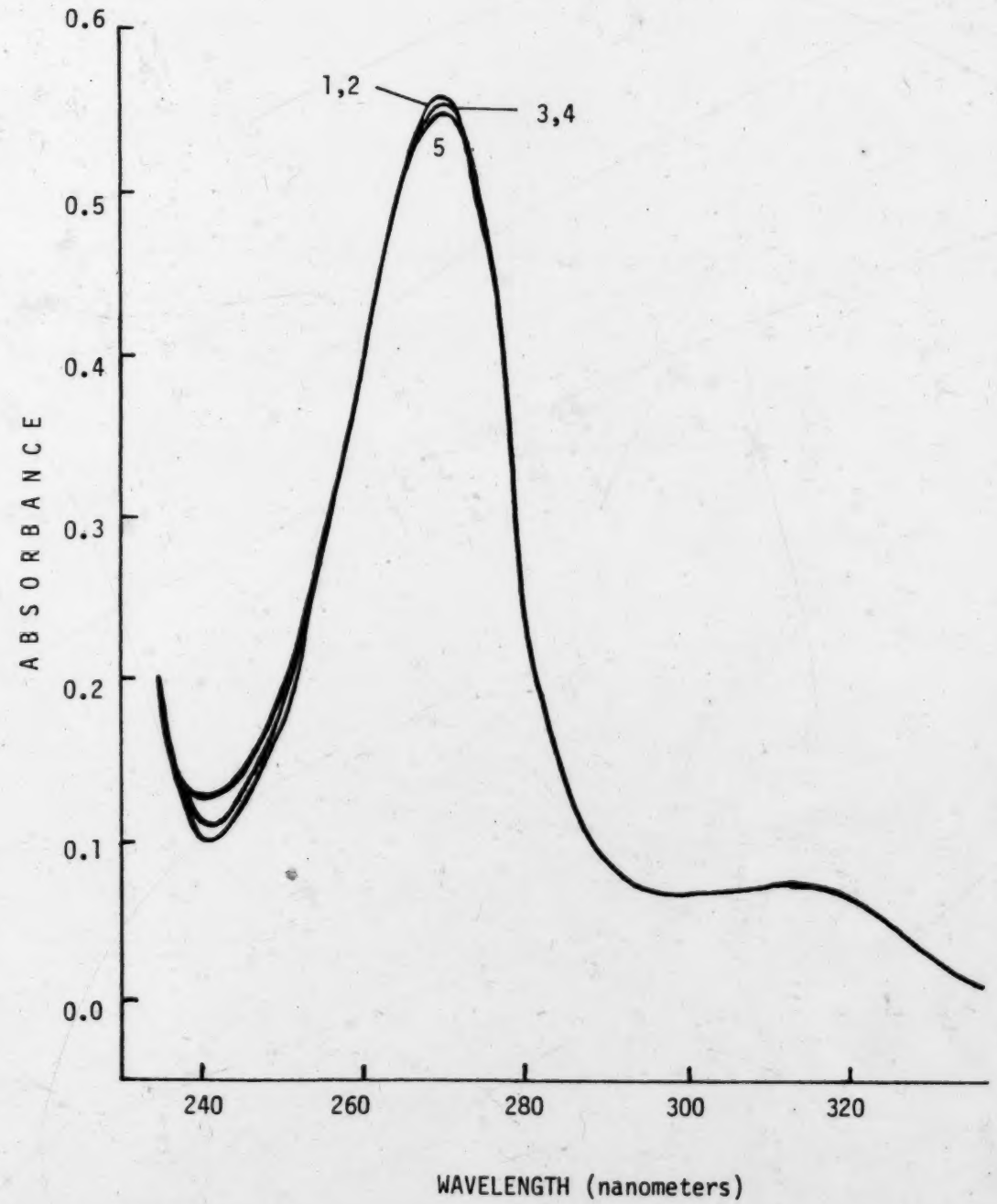


Figure 37. Absorption Spectra of Polythiazide 2 x 10^{-5} M at Various pH Values

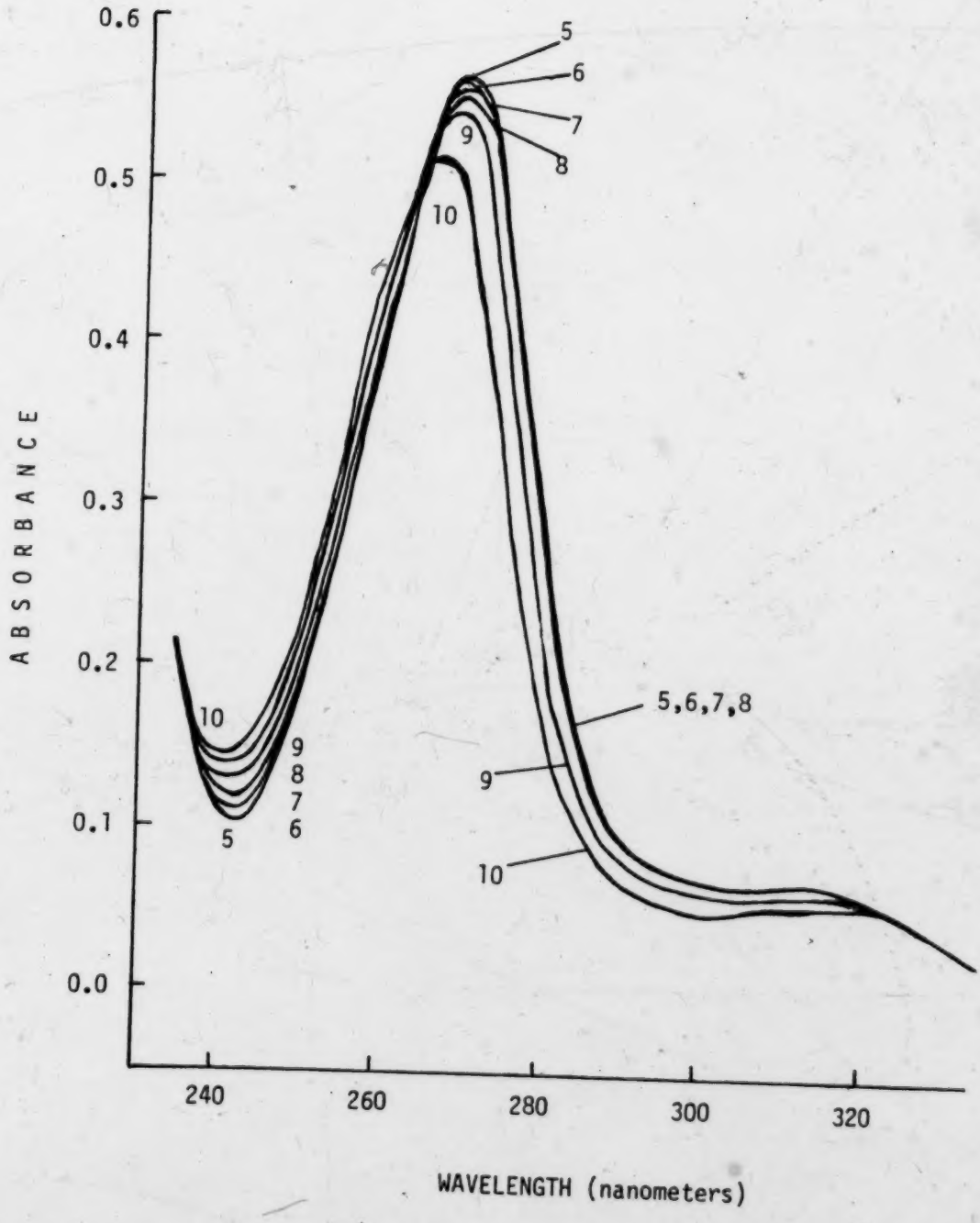
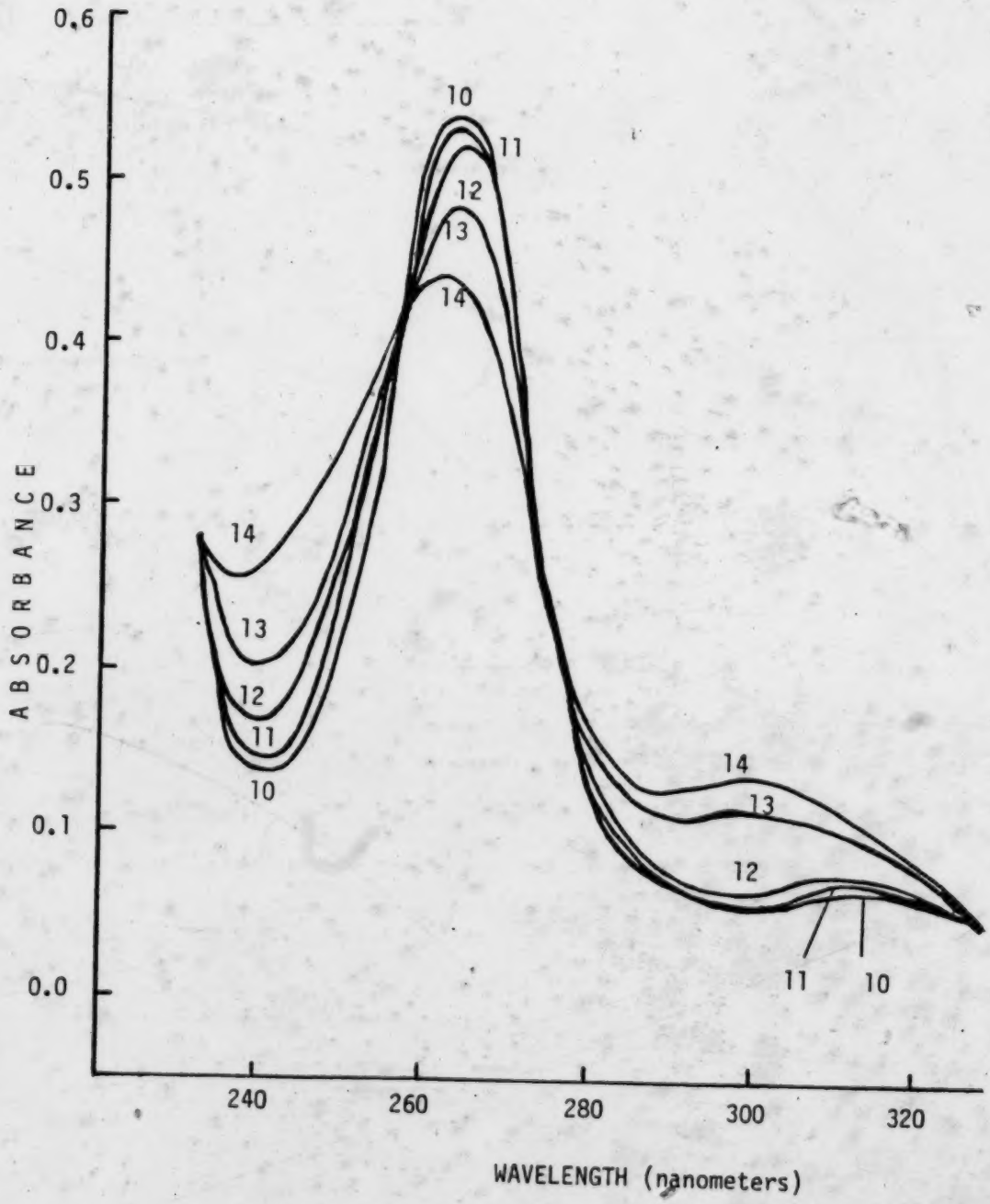


*Figure 38. Absorption Spectra of Polythiazide 2 x 10^{-5} M at Various pH Values

* Obtained under different experimental conditions than the spectra in Figures 36 and 37.



Acid-Base Chemistry

The process of ionization can be expressed by the Brønsted-Lowry theory and the nomenclature as outlined by Albert and Serjeant (20). The dissociation or deprotonation of an acid leads to the production of at least two ionic species, $HA \rightleftharpoons H^+ + A^-$, where H^+ actually represents the hydronium ion in the aqueous solvent, but for simplicity, the term H^+ will be used throughout the subsequent equations. The ionization constant of a monofunctional acid, Ka, can then be expressed in the following terms:

$$K_a^t = \frac{\{H^+\}\ \{A^-\}}{\{HA\}}$$, where { } represents the activity of the particular species present in solution. The ionization of a monofunctional base is depicted as $BH^+ \rightleftharpoons H^+ + B$ and the corresponding ionization constant as:

$$K_a^t = \frac{\{H^+\}\ \{B\}}{\{BH^+\}} .$$

These are thermodynamic ionization constants, K_a^t, and are independent of concentration. When the braces are replaced by square brackets, stoichiometric molar concentrations, rather than activities, are involved and the values obtained are concentration ionization constants, or K_a^c. If only univalent ions are present and the solutions are not stronger than 0.01 M, the concentration ionization constant will correspond closely to the thermodynamic value. At infinite dilution, $K_a^c = K_a^t$.

For convenience, the negative logarithms of the ionization constants are used:

$$pKa = pH + \log \frac{[HA]}{[A^-]} \quad \text{for acids, and}$$

$$pKa = pH + \log \frac{[BH^+]}{[B]} \quad \text{for bases.}$$

These pKa values are then evaluated on a numerical range of 0-14 which is related to the conventional aqueous pH scale. Strong acids and weak bases have low pKa values while weak acids and strong bases have high pKa values.

The pH meter measures hydrogen ion in terms of activity and, therefore, the ionization constant obtained in this manner is composed of a mixture of concentration and activity terms and is a 'mixed' ionization constant, K_a^m. The 'mixed' pKa value is only constant for the concentration of the ionized species present at the particular ionic strength of the sample solution used in the determination and the concentration term must, therefore, be converted to the activity term. The activity of the nonionized species is approximately equal to the concentration.

The conversion of concentration to activity is achieved by the equations:

$$\{A^-\} = [A^-]f_{A^-} \quad \text{for acids, and}$$

$$\{BH^+\} = [BH^+]f_{BH^+} \quad \text{for bases, where } f_{A^-} \text{ and } f_{BH^+} \text{ are the}$$

activity coefficients of the ionized species, and they are usually less than one in numerical value. The activity coefficient, designated

as f_i in general terminology, depends upon the ionic strength of the solution:

$I = 0.5 \Sigma Ciz^2$, where I is the ionic strength, C_i is the concentration of an ion, and z is its valency. The activity coefficient, as related to the ionic strength of the solution, can be defined by the Debye-Hückel equation:

$$-\log f_i = \frac{Az^2 \sqrt{I}}{1 + Ba_i \sqrt{I}},$$

where A and B are constants which vary with the dielectric constant and the temperature of the solvent, and a_i is an ionic size parameter for which an average value of 5×10^{-8} cm is commonly used. Thus, $-\log f_i = \frac{0.512 \sqrt{I}}{1 + 1.5 \sqrt{I}}$ is valid at 25°C if monofunctioning acids or bases, with a valency of one, are under investigation. The calculated term equal to $-\log f_i$ is positive for acids and negative for bases and is used to obtain thermodynamic ionization constants:

$$pK_a^t = pK_a^m \pm \frac{0.512 \sqrt{I}}{1 + 1.5 \sqrt{I}}, \text{ at } 25°C.$$

In addition to obtaining potentiometric pH measurements, the spectrophotometric method of determining ionization constants also involves the reading of the absorbance of the sample, d, in which $d = d_m + d_I$, where both the molecular and the ionized species contribute to the total absorbance. The fraction of each species present varies with the pH of the solution and the pKa is calculated from the pH measurements and absorbances of the samples, together with the absorbances of the purely ionized and the purely molecular species. The fraction of each species, and thus the pKa, is calculated on the

basis of the equations:

$$pK_a^m = pH + \log \frac{d_I - d}{d - d_m} \quad \text{or} \quad pK_a^m = pH + \log \frac{d - d_I}{d_m - d} \quad \text{for acids,}$$

$$\text{and } pK_a^m = pH + \log \frac{d - d_m}{d_I - d} \quad \text{or} \quad pK_a^m = pH + \log \frac{d_m - d}{d - d_I} \quad \text{for bases.}$$

Activity corrections necessary for the buffer salts can be applied to the 'mixed pKa' values using the Debye-Hückel equation, as previously described.

The method is relatively simple as the absorbances of the completely ionized and molecular species can be utilized in calculating their fractions, F_I and F_m, present in the solution. If $d = \varepsilon ct$, then $d = (\varepsilon_I \cdot F_I + \varepsilon_m \cdot F_m)ct$, where ε is the molar absorptivity, c is the concentration, and t is the light pathlength.

The ultraviolet spectrophotometric method has been employed in the determination of the acidity constants of methyclothiazide, polythiazide, and diazoxide (Tables 5-10). Activity corrections were applied and thermodynamic pKa values were calculated. To determine the scatter of the values, first the antilogarithm of each value in the set was taken, then the antilogarithms were averaged, and finally the logarithm of the average was taken. The largest deviation between this value and any value in the set is the scatter (20). It was apparent that, with the data for methyclothiazide (Table 6), the averaging of the antilogarithms did not offer an advantage over the simple averaging of the set of pKa values when determining the scatter. The pK_a^t values reported for methyclothiazide, polythiazide, and diazoxide (Tables 6,8 and 10) are within the range of the average $pK_a^t \pm 0.06$, as determined by the aforementioned method of Albert and Serjeant (20).

DATA FOR METHYCLOTHIAZIDE,
POLYTHIAZIDE, AND DIAZOXIDE

(Tables 5 - 10)

and

(Figure 39)

Table 5. Data for the Determination of the Acidic pKa Value of Methyclothiazide

Methyclothiazide 2 x 10^{-5} M
Analytical Wavelength: 272 nm
Pathlength: 1 cm
Ionic Strength: 0.10 M
Temperature: 22 ± 1°C

d_m (absorbance of molecular species) = 0.537 at pH 7.31
* d_I (absorbance of ionized species) = 0.270 at pH 13.74

	Absorbance			
pH	#1	#2	#3	Average
7.31	0.537	0.537	0.537	0.537
9.00	0.484	0.484	0.484	0.484
9.22	0.430	0.430	0.430	0.430
9.41	0.397	0.397	0.397	0.397
9.59	0.366	0.366	0.366	0.366
9.77	0.330	0.330	0.330	0.330
10.00	0.316	0.317	0.317	0.317
10.18	0.302	0.306	0.304	0.304
13.74	0.275	0.270	0.265	0.270

* At the analytical wavelength of 272 nm the medium effect observed at pH values of 12-14 (Figure 35) is minimized and, therefore, the absorbance at pH 13.74 was used for the ionized species.

Table 6. Determination of the Acidic Dissociation Constant of Methyclothiazide

Methyclothiazide 2 x 10^{-5} M
Analytical Wavelength: 272 nm
Pathlength: 1 cm
Ionic Strength: 0.10 M
Temperature: 22 ± 1°C

$d_I = 0.273$
$d_m = 0.537$

pH	d	$pK_a^m = pH + \log \frac{d-d_I}{d_m-d}$	$pK_a^t = pK_a^m + 0.1092$
9.00	0.484	9.60 *	9.71*
9.22	0.430	9.39	9.50
9.41	0.397	9.36	9.47
9.59	0.366	9.33	9.44
9.77	0.330	9.21 *	9.32 *
10.00	0.317	9.30	9.41
10.18	0.304	9.40	9.51

$pK_a^m = 9.36 \pm 0.06$
$pK_a^t = 9.47 \pm 0.06$

using 5 values in the set (*omitted values)

$pK_a^m = 9.36 \pm 0.24$
$pK_a^t = 9.47 \pm 0.24$

using all 7 values in the set.

Table 7. Data for the Determination of the Acidic pKa Value of Polythiazide

Polythiazide 2×10^{-5} M
Analytical Wavelength: 270 nm
Pathlength: 1 cm
Ionic Strength: 0.01 M
Temperature: 24 ± 1°C

d_m (absorbance of molecular species) = 0.273 at pH 6.80
*d_I (absorbance of ionic species) = 0.208 at pH 10.29

	Absorbance			
pH	#1	#2	#3	Average
6.80	0.272	0.273	0.273	0.273
8.23	0.265	0.265	0.265	0.265
8.47	0.259	0.260	0.261	0.260
8.88	0.250	0.249	0.249	0.249
9.10	0.241	0.240	0.242	0.241
9.25	0.235	0.234	0.233	0.234
9.42	0.227	0.228	0.228	0.228
9.60	0.222	0.223	0.223	0.223
10.29	0.207	0.208	0.209	0.208
13.03	0.196	0.197	0.200	0.198

*At 270 nm the medium effect observed at pH 12-14 is not sufficiently minimized (see Figure 38). This medium effect would not be significant at approximately 275 nm but since this is not the optimum wavelength for measuring absorbance differences (see Figure 37), the absorbance at pH 10.29 was used for the ionized species.

Table 8. Determination of the Acidic Dissociation Constant of Polythiazide

Polythiazide 2×10^{-5} M
Analytical Wavelength: 270 nm
Pathlength: 1 cm
Ionic Strength: 0.01 M
Temperature: 24 ± 1°C

$d_I = 0.208$
$d_m = 0.273$

pH	d	$pK_a^m = pH + \log \frac{d-d_I}{d_m-d}$	$pK_a^t = pK_a^m + 0.0445$
8.23	0.265	9.08	9.13
8.47	0.260	9.07	9.12
8.88	0.249	9.11	9.16
9.10	0.241	9.07	9.11
9.25	0.234	9.07	9.12
9.42	0.228	9.07	9.11
9.60	0.223	9.08	9.12

$pK_a^m = 9.08 \pm 0.03$
$pK_a^t = 9.12 \pm 0.04$

using all 7 values in the set.

Table 9. Data for the Determination of the Acidic pKa Value of Diazoxide

Diazoxide 2×10^{-5} M
Analytical Wavelength: 280 nm
Pathlength: 1 cm
Ionic Strength: 0.01 M
Temperature: 24 ± 1°C

d_m (absorbance of molecular species) = 0.031 at pH 4.98
*d_I (absorbance of ionic species) = 0.101 at pH 10.30

	Absorbance			
pH	#1	#2	#3	Average
4.98	0.031	0.030	0.032	0.031
7.59	0.043	0.041	0.040	0.041
7.81	0.045	0.046	0.048	0.046
8.03	0.053	0.053	0.053	0.053
8.25	0.061	0.062	0.062	0.062
8.45	0.067	0.068	0.067	0.067
8.68	0.078	0.077	0.079	0.078
8.88	0.084	0.083	0.085	0.084
9.10	0.090	0.089	0.091	0.090
10.30	0.100	0.101	0.102	0.101
13.05	0.141	0.140	0.138	0.140

*At 280 nm the medium effect observed at pH 11-14 is not sufficiently minimized (see Figure 4). Since diazoxide is almost completely deprotonated above pH 10, the absorbance at pH 10.30 was used for the ionized species.

Table 10. Determination of the Acidic Dissociation Constant of Diazoxide

Diazoxide 2 x 10^{-5} M
Analytical Wavelength: 280 nm
Pathlength: 1 cm
Ionic Strength: 0.01 M
Temperature: 24 ± 1°C

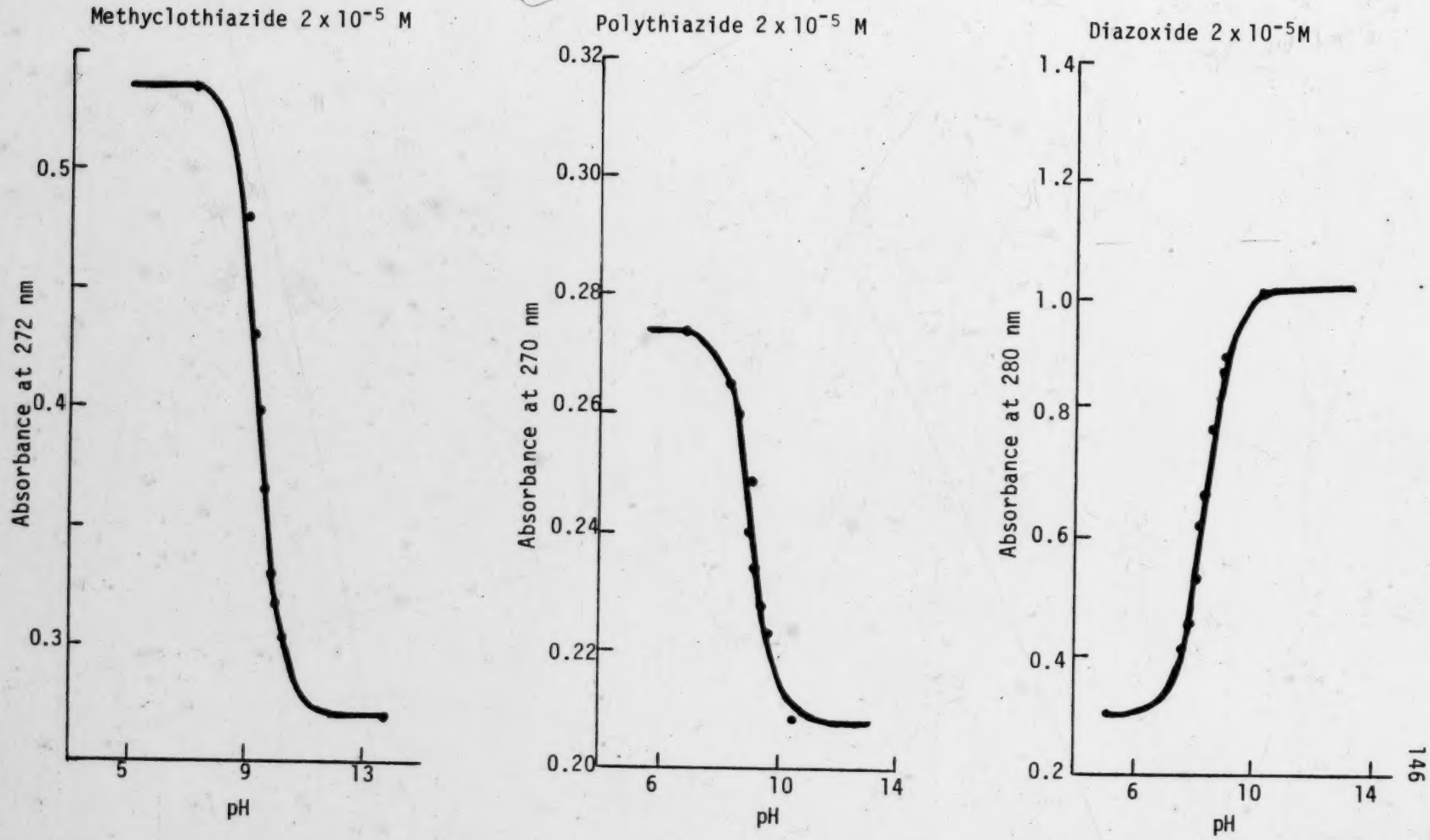
pH	d	$pK_a^m = pH + \log \frac{d_I - d}{d - d_m}$	$pK_a^t = pK_a^m + 0.0445$
7.59	0.041	8.37	8.41
7.81	0.046	8.37	8.42
8.03	0.053	8.37	8.41
8.25	0.062	8.35	8.39
8.45	0.067	8.42	8.47
8.68	0.078	8.37	8.41
8.88	0.084	8.39	8.43
9.10	0.090	8.37	8.42

$pK_a^m = 8.38 \pm 0.04$

$pK_a^t = 8.42 \pm 0.05$

using all 8 values in the set.

Figure 39. Graphical Representation of Absorbance-pH Data of Methyclothiazide, Polythiazide and Diazoxide



The theoretical lines in Figure 39 were calculated from the equation $\varepsilon = \frac{\varepsilon_m[H^+] + \varepsilon_I K_a}{[H^+] + K_a}$ to fit the experimental points. The terms ε_m and ε_I represent the molar absorptivities of the molecular and ionized species respectively and ε is the observed molar absorptivity at each pH value.

Overlapping Ionization Constants

The relationship between the ionized and the molecular species in UV spectrophotometry becomes more complex when the ionizations of two functional groups in the compound overlap. The acidity constants are considered to be overlapping when they lie within three pKa units of one another. The absorbances of the purely molecular species and one of the ionic forms can usually be measured without difficulty but the absorbance of the intermediate ionic species may not be so readily obtainable. This is due to the fact that the ionization of the second functional group begins before the titration of the first group has been completed.

The general nomenclature of Albert and Serjeant (20) can be applied to all polyprotic compounds and, therefore, the benzothiadiazines with overlapping pKa values can also be examined in this manner:

$$\text{diprotonated} \overset{Ka_1}{\rightleftharpoons} \text{monoprotonated} + H^+ \overset{Ka_2}{\rightleftharpoons} \text{nonprotonated} + H^+$$

If Ka_1 and Ka_2 are the acid dissociation constants of the benzothiadiazines then the diprotonated form is the molecular species and the nonprotonated form is the completely ionized species, with respect to the acidic ionization processes only.

The observed or measured molar absorptivity is ε at a given pH and, therefore, $\varepsilon = \varepsilon_C \cdot F_C + \varepsilon_m \cdot F_m + \varepsilon_A \cdot F_A$ (equation 1), where ε_C, ε_m and ε_A are the molar absorptivities of the diprotonated, monoprotonated, and nonprotonated species, respectively, while F_C, F_m and F_A are the fractions of each of these species present in the solution.

The fractions of each of these species can be further defined as $F_C = \frac{[H^+]^2}{D}$, $F_m = \frac{K_1[H^+]}{D}$, and $F_A = \frac{K_1K_2}{D}$, where the denominator, D, is $[H^+]^2 + K_1[H^+] + K_1K_2$. Substituting these fractions into equation 1 and multiplying throughout by D gives the formula:

$$[H^+]^2(\varepsilon - \varepsilon_C) + K_1[H^+](\varepsilon - \varepsilon_m) + K_1K_2(\varepsilon - \varepsilon_A) = 0,$$

which can be rewritten as:

$$\frac{[H^+]^2}{K_1}\ \frac{(\varepsilon - \varepsilon_C)}{(\varepsilon - \varepsilon_A)} + K_2 = -[H^+]\frac{(\varepsilon - \varepsilon_m)}{(\varepsilon - \varepsilon_A)} \quad \text{(equation 2).}$$

Upon examining equation 2 it is apparent that the three variables are ε_m, K_1 and K_2. To evaluate these terms the absorbance-pH data, obtained at 0.2 pH unit intervals between the diprotonated and the nonprotonated species, is divided into two sections. The ε_1 data covers the ionization of the stronger acidic group, whereas the ε_2 data covers the ionization of the weaker group.

When inspecting the pH region of the ionization of the stronger group, one can assume that the predominant species in solution will be the diprotonated and the monoprotonated forms, therefore, the fraction of the nonprotonated species that is present will be small, though not negligible. The substitution into equation 1 is performed in the same manner as before but leaving the $\varepsilon_A F_A$ portion undefined:

$$\varepsilon_1 D = \varepsilon_C [H^+]^2 + \varepsilon_m K [H^+] + \varepsilon_A F_A D.$$

This form of the equation is then rearranged to:

$$(1 + \frac{K_2}{[H^+]})(\varepsilon_1 - \varepsilon_A F_A) = \varepsilon_m + \frac{[H^+]}{K_1}(\varepsilon_C - \varepsilon_1 + \varepsilon_A F_A) \text{(equation 3a).}$$

Similar adjustments to equation 1 are made for the second ionization process, where the fraction of the diprotonated species present is considered to be small, but not negligible:

$$\varepsilon_2 D = \varepsilon_m K_1 [H^+] + \varepsilon_A K_1 K_2 + \varepsilon_C F_C D,$$

and a rearrangement of this equation yields:

$$(1 + \frac{[H^+]}{K_1})(\varepsilon_2 - \varepsilon_C F_C) = \varepsilon_m + \frac{K_2}{[H^+]} (\varepsilon_A - \varepsilon_2 + \varepsilon_C F_C) \text{(equation 3b).}$$

Equations 3a and 3b are used to obtain estimates of the molar absorptivity of the monoprotonated species. The process is initiated by setting the terms $(1 + \frac{K_2}{[H^+]})$ and $(1 + [H^+]/K_1)$ equal to unity and the terms $\varepsilon_A F_A$ and $\varepsilon_C F_C$ equal to zero.

For the absorbance-pH data of the first ionization process, the mean ε_m value obtained from equation 3a is substituted into equation 2, and K_1 and K_2 values are calculated using all of the data uniformly. These K_1 and K_2 values are then used to calculate corrections for the $(1 + K_2/[H^+])$ and $\varepsilon_A F_A$ terms, which are substituted into equation 3a to calculate a new ε_m value. This new ε_m value is used in equation 2 and the aforementioned process is continued reiteratively until no significant difference between successive K_1 and K_2 values is observed.

The absorbance-pH data of the second ionization process is treated in the same manner using equations 3b and 2, and the corrections are

calculated for the $(1 + [H+]/K_1)$ and $\varepsilon_c F_c$ terms. The final ε_m values from the two sets of data are averaged and the average value is used in equation 2 for the calculation of the pka_1 and pka_2 values. This final step is carried out without reference to the iteration process.

Activity corrections have been applied to equations 2, and 3a and b, thereby accounting for the ionic strength of the buffer salts employed in the experiment. Equation 2 can be written in the form yielding x and y coordinates, $y = K_2 + \frac{x}{K_1}$, where the derived K_1 and K_2 values are 'mixed constants', a combination of concentration and activity terms.

The dissociation of dibasic acids can be represented as:

$$H_2A \overset{K_1}{\rightleftharpoons} H^+ + HA^- \overset{K_2}{\rightleftharpoons} H^+ + A^{-2}$$

and activity corrections involve the activity coefficients of the ionized species so that $K_1^t = K_1^m \cdot f_{HA^-}$ and $K_2^t = \frac{K_1^m \cdot f_{A^{-2}}}{f_{HA^-}}$.

Calculation of the correction factors for activity is achieved by the Debye-Hückel equation, as previously described for monofunctioning acids and bases, and the thermodynamic acidity constants are expressed as:

$$K_1^t = \frac{K_1^m}{10^{(0.512FS)}} \text{ and } K_2^t = \frac{K_2^m}{10^{(1.536FS)}} \text{ at } 25°C, \text{ where } FS = \frac{\sqrt{I}}{1+1.5\sqrt{I}}.$$

The rearrangement of these equations to $K_1^m = K_1^t \cdot 10^{(0.512FS)}$ and $K_2^m = K_2^t \cdot 10^{(1.536FS)}$, and the substitution of these terms into the modified version of equation 2, yields the corrected form of the equation for dibasic acids:

$$\frac{y}{10^{(1.536\ FS)}} = \frac{x}{K_1^t \cdot 10^{(2.048\ FS)}} + K_2^t \quad .$$

If the expressions A, A_1, and A_2 define $10^{(0.512\ FS)}$, $10^{(1.536\ FS)}$, and $10^{(2.048\ FS)}$, respectively, the simplified form of modified equation 2 is $\frac{y}{A_1} = \frac{x}{K_1^t A_2} + K_2^t$, where the y-intercept is K_2^t and the slope is $\frac{1}{K_1^t}$.

The calculations required for overlapping ionization constants can be incorporated into a computer program that accepts absorbance-pH input data and gives pKa_1 and pKa_2 values as output. Albert and Serjeant (20) have published such a program in FORTRAN IV which, in fact, performs the appropriate calculations. This program was employed in the determination of the overlapping acidic ionization constants of selected benzothiadiazines and the computer input data was derived from the linear portions of the plots of absorbance versus pH (Figures 40-48). The data selection was made from the linear sections only because these are the regions of the graphs where the major portions of the ionizations take place. In certain instances the region of the second acidic ionization process was not clearly defined because the difference between the molar absorptivities of the monoprotonated and the nonprotonated species is extremely small. Such is the situation with althiazide (Figure 43) and cyclopenthiazide (Figure 48), for which the region of the second dissociation had to be estimated from a combination of absorbance-pH data and UV spectral-pH data. This estimation was necessary to obtain reasonable computer input data so that a pKa_1 value could be calculated for these particular benzothiadiazines.

The first difficulty encountered with the FORTRAN IV computer program was the failure of the K_1 and K_2 values to converge. In an attempt to eliminate this problem, a new computer program in FOCAL 11 was written without reference to the FORTRAN IV program. The new program included activity corrections for 20°C as well as for 25°C. The problems previously experienced appeared to be alleviated, but only for 6 of the 9 benzothiadiazines examined. Comparisons between the ε_m, K_1 and K_2 values obtained from the ε_1 and ε_2 data are displayed in the computer print-out (Tables 20-29).

Bendroflumethiazide (Table 27) exhibited convergence only for the ε_1 set of data and, therefore, an ε_{m_1} value was calculated. The erratic computer output for the nonconvergent ε_2 data was then scrutinized for meaningful K_1, K_2 and ε_{m_2} values and when 0.164099×10^5 was utilized as the ε_{m_2} value, and averaged with the ε_{m_1} value, a pKa_1 of 9.0 and a pKa_2 of 10.5 were manually calculated. These experimental calculations yielded pKa values which were in good agreement with those of approximately 8.8 and 10.2 estimated by visually inspecting the graph of absorbance versus pH of bendroflumethiazide (Figure 46). Although the manually calculated pKa_2 value is not reliable, the stratagem was a necessary step in obtaining a reasonably accurate pKa_1 value.

The scatter of the points along the linear portions of the plots of absorbance versus pH was initially suspected to be the reason for the lack of convergence. A least squares analysis becomes difficult to perform since the computer perceives the possibility of more than one 'best fit' line and an oscillation, which may also be dependent upon the degree of scatter, between the possible values results.

Cyclopenthiazide and cyclothiazide did not exhibit convergence

for either of the ε_1 or the ε_2 sets of data and, therefore, their pKa values could not even be manually calculated, since the reiterative correction process must be completed before a useful ε_{m_1} value can be obtained. The erratic computer output cannot always be attributed to the scatter of the absorbance-pH data, as may appear to be the problem with cyclopenthiazide (Figure 48), because the same type of graphical representation of the absorbance-pH data of cyclothiazide (Figure 47) exhibits minimal scatter.

Other factors that could, in part, be responsible for the non-convergence are the small difference between the molar absorptivities of the monoprotonated and the nonprotonated species and the effect of the strongly basic buffers of 0.1 M ionic strength used in determining the weaker of the acidic dissociation constants. The former would be expected to be the major contributing factor since the chosen graphical points are few in number, and perhaps also scattered, and the straight line fitting process may not achieve a concrete value. With the possibility of more than one straight line, the y-intercept and the slope will vary so drastically upon each recalculation that no final value will be achieved. This occurrence is often due to one or more of the chosen points being equal to, or almost equal to, the value of either the molar absorptivity of the diprotonated species (ε_C) or that of the nonprotonated species (ε_A). In either circumstance a value of zero or an insignificant difference will result which may not be acceptable to the computer and so erroneous K_1, K_2 and ε_m values, that do not and never will converge, are eventually obtained.

Another problem that was encountered with the computer program was

the calculation of negative logarithms. Althiazide, flumethiazide, and chlorothiazide presented this type of difficulty and, again, the predicament arises from the choice of input data. For flumethiazide and chlorothiazide, manual calculations provided credible pKa_1 and pKa_2 values, respectively, but the signs of the modified x- and y-values and the K-values are responsible for the negative logarithms. For example, as a result of the negative modified x-values, the positive modified y-values, and a K_2 value of lower numerical value than the modified y-values obtained for chlorothiazide, the pKa_1 value for this compound could not be calculated since the logarithm of a negative number is mathematically undefined and is considered to be zero. This problem is inherent in the methodology and will occur if the input data does not satisfy the numerical requirement of the computer.

In general, the computer method would be useful if a program could be devised that would permit operator intervention when oscillation, due to the precision factor required by the instrument, begins. This would be the ideal situation but the computer must be adequately programmed while taking into account the practical aspects of such procedures. The PDP 11-05 Minicomputer used in this experiment requires precision to the sixth post-decimal digit and this requirement can affect the convergence check. If, perhaps, the successive K_1 and K_2 values obtained are not identical to the sixth digit, the computation cannot proceed past this point. A simple average, however, or even the existing values, could be extremely accurate and very useful for further calculation. To alleviate this problem the conditions of the

program can be relaxed to a lower precision requirement by the insertion of a new command into the already existing computer program.

The remedy for the oscillation or lack of convergence may, itself, produce errors. A lack of precision would arise if too few identical digits are required by the program for the convergence check. For this reason it would be much more desirable to have an optional command which would permit the computer to accept operator inspected values but this ideal mechanism is not a practical solution in computer programming.

The ultraviolet spectrophotometric experimental procedure itself presents problems since there is little difference between the molar absorptivities of the monoprotonated and the nonprotonated species of the benzothiadiazines. This difficulty can be attributed to the site of the second acidic deprotonation which exerts only a minor electronic effect on the chromophore. The result is a minimum shift in wavelength and a limited degree of change in absorbance intensity. The site of this deprotonation has been postulated to be at the exocyclic sulfonamido group (7, 9, 19) because this functional group is more remote from the ring system than the cyclic sulfonamido group and it is in a similar chemical environment in all of the benzothiadiazines. Therefore, the deprotonation of the exocyclic sulfonamido group should effect only a minor redistribution of electrons within the molecule of the particular benzothiadiazine.

The almost insignificant differences between the molar absorptivities accounts for the scattered absorbance-pH data (Figures 40-48), which is especially evident in the region of the second acidic ioniza-

tion process. Due to the insignificant absorbance differences, the values obtained for the second pKa may be invalid but the values obtained for the first pKa (Table 30) appear to be consistent with the approximate values that are procurable from the UV spectra (Figures 14-32) and the values that can be estimated from the plots of absorbance versus pH (Figures 40-48), for each benzothiadiazine. Furthermore, these pKa_1 values, when compared to the literature values in Table 31, are in near agreement with the previously reported values.

The high pKa_2 values of greater than 11 that were obtained may also be artefacts of the extremely basic buffer solutions and their high ionic strength of 0.1 M. This results in a numerically larger activity correction factor being added to the 'mixed constant' when thermodynamic values are being calculated. The pKa_2 values of chlorothiazide and flumethiazide, determined at an ionic strength of 0.01 M, were graphically estimated from Figures 44 and 45 and were found to be 9.7 and 9.4, respectively. These values seem to fall within the expected range, even after activity corrections have been applied.

The effect of the organic solvent, in semiaqueous solutions, on the dissociation constants of benzothiadiazines has been examined by Whitehead et al. (7,8). Experimentation with chlorothiazide and some of its analogs revealed that the first acidic ionization constants were usually unaffected by the 66% dimethylformamide solvent system but the second acidic ionization constants were elevated to values which averaged at approximately 12.5. The results of Novello and Sprague (78) indicated that 30% methanol had little effect on the pKa_2 of cyclothiazide

since the value of 10.7 estimated from the midpoint of the line of the graph of absorbance versus pH (Figure 47) is in near agreement with their value of 10.5. Furthermore, the extrapolation technique of Moskalyk et al. (80) has yielded pKa_1 values, for various benzothiadiazines, that approximate those which were determined by other techniques (Table 31).

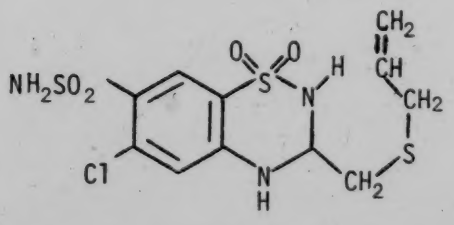
The values of the first acidic ionization constants of the thiazides and hydrothiazides, when determined by ultraviolet spectrophotometry, appear to be reliable but the values of the second ionization constants may be invalid. Mollica et al. (13) also experienced difficulty in determining the pKa_2 value of hydrochlorothiazide, therefore, one would expect this parameter of other benzothiadiazines, with an even smaller difference between the molar absorptivities of the monoprotonated and the nonprotonated species, to be difficult to determine. This may explain the unusually high figures of 11.3 , 12.0 and 11.2 obtained for hydrochlorothiazide, hydroflumethiazide, and trichloromethiazide, respectively. If the UV absorbption spectra and the absorbance-pH data of the compounds are examined, it can be assumed that these pKa_2 values should be below 11 and the values estimated from the graphs of absorbance versus pH (Figures 40-42) would be more realistic.

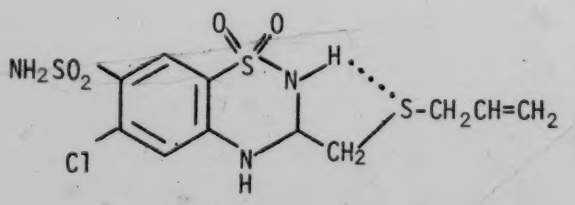
The values for the first pKa range from 6.0 -9.5 , with the thiazides yielding the lower values and the hydrothiazides yielding the higher values. The first acidic dissociation constants exhibit greater variation than the second ionization constants and this effect can be attributed to the substituents at the 3-position. The fact that the pKa_2 values of the benzothiadiazines are similar is

predictable if the site of deprotonation is assumed to be the exocyclic sulfonamido group. Since the only variation in the neighbouring substituents of the exocyclic sulfonamido group is the presence of either a chlorine or a trifluoromethyl group at the 6-position, one would expect the pKa_2 values to be similar.

Hydrothiazides, such as hydrochlorothiazide and hydroflumethiazide, with a hydrogen at the 3-position appear to behave as weaker acids since this hydrogen has little, if any, electronic effect on the cyclic sulfonamido group. The replacement of the hydrogen with a benzyl group, as in bendroflumethiazide, decreased the pKa_1 value only slightly since the electron withdrawing effect of the phenyl group is diminished by the intervening methylene group.

The 3-substituents of trichloromethiazide and althiazide probably exert an electron withdrawing effect on the cyclic sulfonamido group, producing stronger acids. The long chain substituent of althiazide may form a more stable pseudo-five or-six membered ring through hydrogen bonding at the 2-position:



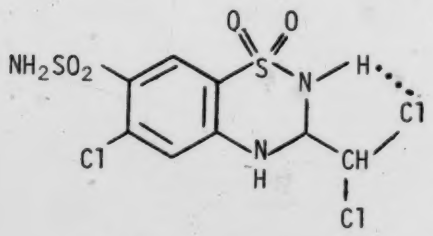


pseudo-six membered or pseudo -five membered ring systems

This hydrogen is attracted to either of the nucleophilic vinyl or sulfur components.

A similar hypothesis can be expressed for trichloromethiazide

since its 3-substituent contains nucleophilic chlorine atoms:



trichloromethiazide

In contrast, the cyclopentyl and norbornylenyl substituents, as contained in cyclopenthiazide and cyclothiazide, contribute electrons to the acidic functional group. The effect of this is to weaken the acidic property of the compounds.

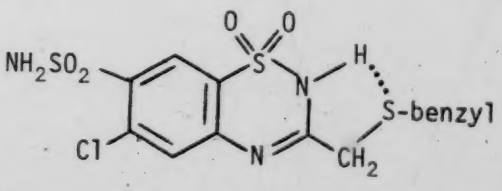
The effect of substitution at the 3-position on the pKa_1 values of the thiazides could not be studied in predominantly aqueous solutions because benzthiazide, the available reference compound with a 3-substituent, precipitated from the solution when water was added to an ethanolic stock solution of the drug. The experimental conditions for ultraviolet spectrophotometry in this investigation required a 0.2% ethanolic sample solution and any greater concentrations of organic solvent were not acceptable.

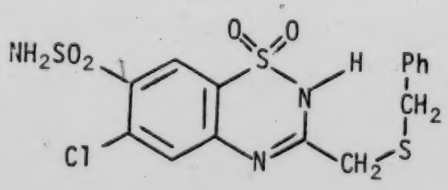
Whitehead et al. (7) studied 3-substituted thiazides in 66% dimethylformamide and, as they observed an almost negligible effect of the organic solvent on the first acidic dissociation constant, their conclusions may also be valid for thiazides in predominantly aqueous solvents:

> "the ionization of the cyclic sulfonamide is effected by the 3-substituent. Cycloalkylmethyl groups decrease the ionization. A double bond in the ring of the cycloalkylmethyl group appears to lessen this effect if the double bond is in a position nearest the methyl carbon attached to the ring. Introduction of an electronegative element, i.e., oxygen or sulfur, between the methyl

> carbon and the cycloalkyl ring practically nullifies the effect of the cycloalkyl group. The cyclic sulfonamide is a much stronger acid, pKa 5.4 and 6.3 when the 3-substituent is arylsulfonylmethyl and aryloxymethyl." (7)

The UV absorption spectra of benzthiazide (Figures 11-13) indicate an approximate pKa_1 value of 6. This suggests that benzthiazide is a slightly stronger acid than chlorothiazide and that it is of comparative acidity to flumethiazide. Although the resolution of the overlapping pKa values of benzthiazide was not achieved under the experimental conditions of this investigation, the pseudo-five or -six membered ring theory could also apply to this compound.





benzthiazide

The theory is applicable because the same type of effects caused by the 3-substituents of the hydrothiazides should also be observed with thiazides substituted at the 3-position.

The dissociation of the acidic hydrogens of methyclothiazide and polythiazide occurs at the proposed site of the dissociation of the second acidic hydrogen of the thiazides and hydrothiazides. The pKa_1 values of the N^2-methylhydrothiazides are approximately one pKa unit lower than the corresponding pKa_2 values of the hydrothiazides. A possible explanation is the effect of the 3-substituent on the

exocyclic sulfonamido group. Although the 3-substituent is at a site remote from the ionizing group, NMR evidence has indicated that a significant shift of the protons at carbon 3 occurs when the acidic sulfonamido hydrogen is titrated with base. This NMR evidence suggests that the 3-substituents of the benzothiadiazines have a considerable influence on both of the acidic protons.

The acidic exocyclic sulfonamido hydrogen of the hydrothiazides is leaving a negatively charged ion and this anion may be a weaker acid than the N^2-methylhydrothiazides. This factor would tend to cause the pKa_2 of the hydrothiazides to be higher than the corresponding pKa_1 of the N^2-methylhydrothiazides.

In summary, the electronic nature of the substituent at the 3-position of a benzothiadiazine appears to be primarily responsible for the increase or decrease in the acidity of the functional groups.

ABSORBANCE-pH DATA

(Figures 40 - 48)
and
(Tables 11 - 19)

The theoretical lines in Figures 40-48 were calculated from the equation

$$\varepsilon = \frac{\varepsilon_C[H^+]^2 + \varepsilon_m K_1[H^+] + \varepsilon_A K_1 K_2}{[H^+]^2 + K_1[H^+] + K_1 K_2}$$

to fit the experimental points. The terms ε_C, ε_m and ε_A represent the molar absorptivities of the diprotonated, monoprotonated, and nonprotonated species, respectively, and ε is the observed molar absorptivity at each pH value. Absorbance and molar absorptivity are interchangeable in this equation as long as the drug concentration is not altered.

The computer program did not provide pKa_1 and pKa_2 values for all of the benzothiadiazines under investigation. The theoretical lines for these particular compounds were calculated using estimated values of pKa_1 and pKa_2. For althiazide and flumethiazide the pKa_2 values were estimated to be 11 and 9, respectively (Figures 43 and 44) and a pKa_1 value of 6.81 (an average of the literature values in Table 1) was used for chlorothiazide. The theoretical lines calculated with these estimated pKa values are represented by a dashed line (- - -).

The theoretical lines for cyclothiazide and cyclopenthiazide could not be calculated since the computer program did not provide ε_m, pKa_1 or pKa_2 values for either of these benzothiadiazines (Figures 47 and 48).

Table 11. Data for the Determination of the Overlapping pKa Values of Hydrochlorothiazide

Hydrochlorothiazide 2×10^{-5} M
Analytical Wavelength: 270 nm
Pathlength: 1 cm
Ionic Strength: 0.10 M
Temperature: 22 ± 1°C

	Absorbance			
pH	#1	#2	#3	Average
5.47	0.357	0.358	0.357	0.357
5.74	0.379	0.379	0.379	0.379
5.94	0.360	0.360	0.363	0.361
6.15	0.352	0.352	0.355	0.353
6.40	0.358	0.357	0.356	0.357
6.53	0.423	0.424	0.429	0.425
6.76	0.357	0.354	0.355	0.355
6.95	0.366	0.364	0.365	0.365
7.14	0.361	0.362	0.363	0.362
7.35	0.358	0.357	0.358	0.358
7.55	0.365	0.366	0.364	0.365
7.76	0.365	0.366	0.364	0.365
7.98	0.373	0.370	0.362	0.368
8.20	0.352	0.347	0.348	0.349
8.46	0.351	0.351	0.355	0.352
8.73	0.341	0.344	0.345	0.343
8.75	0.372	0.372	0.372	0.372
8.94	0.340	0.339	0.338	0.339
9.09	0.328	0.329	0.329	0.329
9.29	0.320	0.320	0.328	0.323
9.49	0.299	0.299	0.301	0.300
9.67	0.275	0.287	0.290	0.284
9.82	0.280	0.245	0.280	0.268
10.02	0.269	0.262	0.265	0.265
10.28	0.259	0.260	0.264	0.261
10.41	0.270	0.270	0.271	0.270
10.62	0.270	0.271	0.270	0.270
10.77	0.272	0.272	0.272	0.272
10.96	0.270	0.270	0.270	0.270
10.97	0.281	0.282	0.280	0.281
11.21	0.277	0.278	0.276	0.277
11.31	0.296	0.296	0.296	0.296
11.58	0.296	0.294	0.295	0.295
11.79	0.303	0.306	0.306	0.305
11.95	0.284	0.291	0.302	0.292

Figure 40. Graphical Representation of Absorbance-pH Data of Hydrochlorothiazide 2 x 10^{-5} M

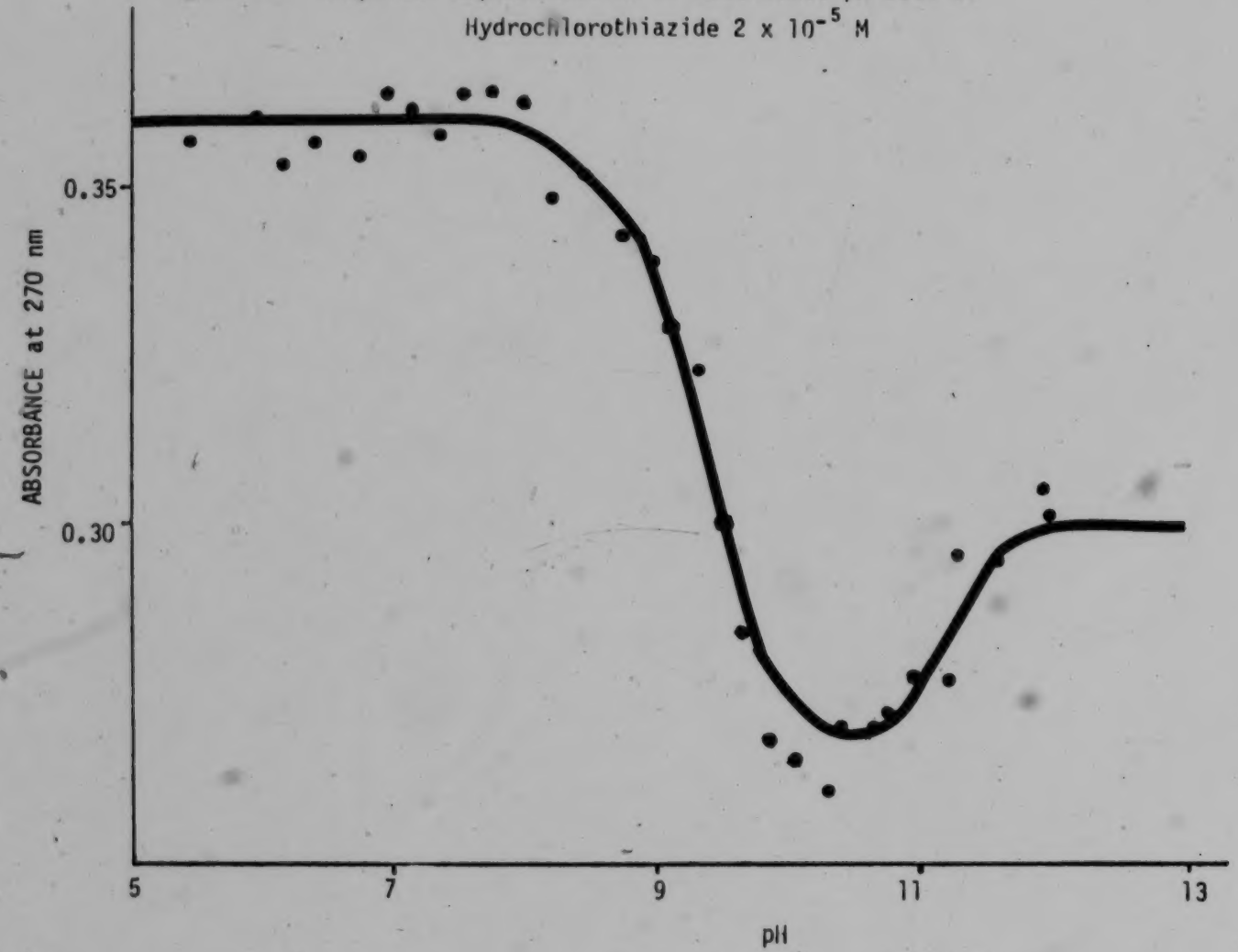


Table 12. Data for the Determination of the Overlapping pKa Values of Hydroflumethiazide

Hydroflumethiazide 2×10^{-5}M
Analytical Wavelength: 270 nm
Pathlength: 1 cm
Ionic Strength: 0.10 M
Temperature: 22 ± 1°C

	Absorbance			
pH	#1	#2	#3	Average
6.51	0.346	0.345	0.346	0.346
6.75	0.374	0.376	0.375	0.375
6.94	0.379	0.381	0.383	0.381
7.16	0.350	0.350	0.339	0.345
7.37	0.353	0.353	0.353	0.353
7.52	0.325	0.323	0.337	0.328
7.73	0.353	0.352	0.352	0.352
7.95	0.359	0.358	0.360	0.359
8.16	0.354	0.353	0.353	0.353
8.44	0.350	0.350	0.347	0.349
8.72	0.329	0.331	0.330	0.330
9.09	0.309	0.309	0.310	0.309
9.27	0.309	0.305	0.304	0.306
9.47	0.287	0.294	0.292	0.291
9.64	0.286	0.287	0.287	0.287
9.78	0.285	0.270	0.279	0.278
9.97	0.272	0.272	0.275	0.273
10.21	0.263	0.263	0.270	0.265
10.38	0.266	0.266	0.267	0.266
10.55	0.273	0.274	0.272	0.273
10.71	0.272	0.273	0.271	0.272
10.92	0.278	0.278	0.279	0.278
10.93	0.277	0.276	0.275	0.276
11.22	0.290	0.293	0.292	0.292
11.36	0.292	0.291	0.290	0.291
11.58	0.291	0.292	0.292	0.292
11.89	0.310	0.310	0.309	0.310
11.93	0.304	0.303	0.304	0.304
12.21	0.290	0.291	0.290	0.290
12.35	0.308	0.309	0.308	0.308
12.62	0.318	0.320	0.320	0.319
12.82	0.320	0.323	0.320	0.321

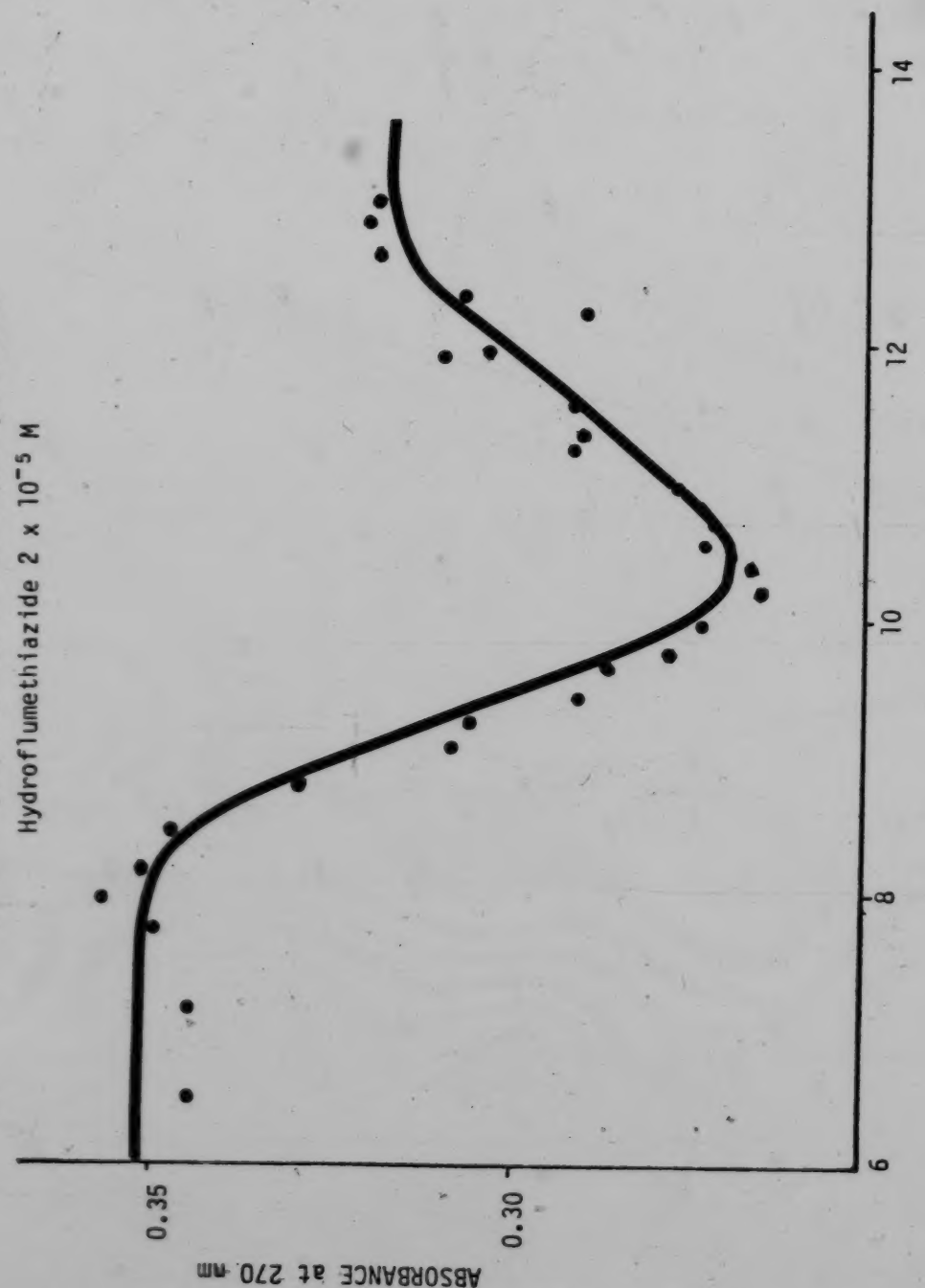


Figure 41. Graphical Representation of Absorbance-pH Data of Hydroflumethiazide 2×10^{-5} M

Table 13. Data for the Determination of the Overlapping pKa Values of Trichloromethiazide

Trichloromethiazide 2×10^{-5} M
Analytical Wavelength: 270 nm
Pathlength: 1 cm
Ionic Strength: 0.10 M
Temperature: 24 ± 1°C

	Absorbance			
pH	#1	#2	#3	Average
5.55	0.401	0.400	0.400	0.400
5.82	0.389	0.389	0.391	0.390
6.01	0.390	0.389	0.390	0.390
6.24	0.375	0.374	0.375	0.375
6.44	0.388	0.386	0.387	0.387
6.63	0.375	0.375	0.375	0.375
6.84	0.375	0.375	0.372	0.374
7.03	0.361	0.360	0.359	0.360
7.24	0.355	0.355	0.355	0.355
7.43	0.339	0.340	0.341	0.340
7.62	0.334	0.334	0.334	0.334
7.84	0.316	0.315	0.314	0.315
8.05	0.303	0.304	0.304	0.304
8.21	0.310	0.311	0.312	0.311
8.50	0.293	0.293	0.293	0.293
8.77	0.287	0.287	0.288	0.287
8.79	0.295	0.295	0.294	0.295
9.00	0.296	0.296	0.299	0.297
9.13	0.293	0.293	0.293	0.293
9.35	0.285	0.289	0.288	0.287
9.54	0.295	0.293	0.295	0.294
9.72	0.287	0.288	0.284	0.286
9.80	0.291	0.289	0.290	0.290
9.80	0.310	0.310	0.310	0.310
9.99	0.300	0.300	0.301	0.300
10.23	0.301	0.300	0.301	0.301
10.31	0.298	0.297	0.299	0.298
10.70	0.304	0.304	0.303	0.304
10.90	0.311	0.309	0.308	0.309
10.93	0.312	0.311	0.305	0.309
11.10	0.315	0.314	0.314	0.314
11.22	0.325	0.325	0.325	0.325
11.42	0.324	0.325	0.320	0.323
11.62	0.320	0.324	0.321	0.322
11.96	0.332	0.333	0.336	0.334

Figure 42. Graphical Representation of Absorbance-pH Data of Trichloromethiazide 2 x 10^{-5} M

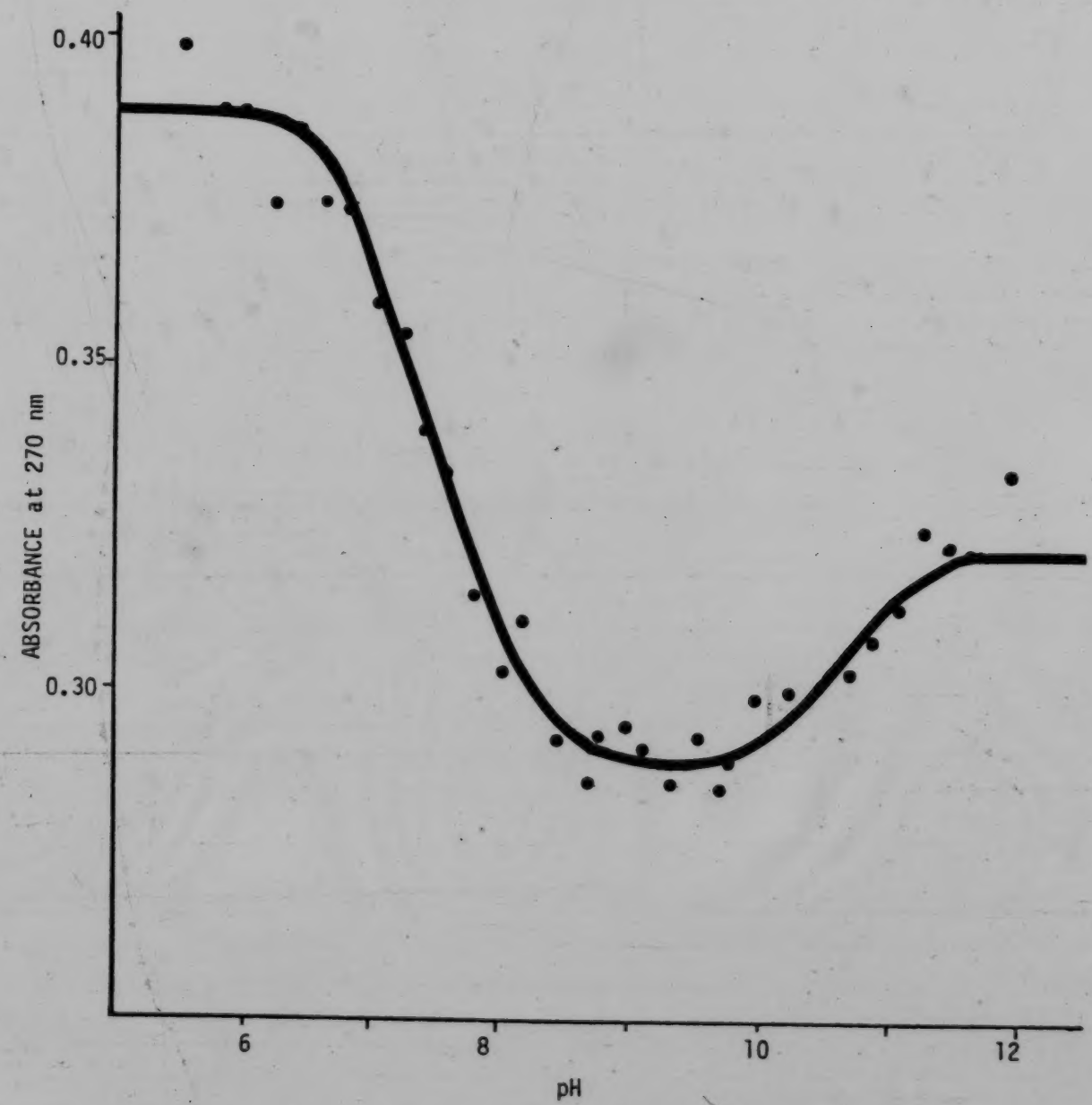


Table 14. Data for the Determination of the Overlapping pKa Values of Althiazide

Althiazide 2 x 10^{-5} M
Analytical Wavelength: 270 nm
Pathlength: 1 cm
Ionic Strength: 0.10 M
Temperature: 23 ± 1°C

	Absorbance			
pH	#1	#2	#3	Average
5.74	0.387	0.387	0.387	0.387
5.94	0.367	0.369	0.369	0.368
6.22	0.365	0.365	0.365	0.365
6.42	0.368	0.365	0.366	0.366
6.60	0.379	0.378	0.370	0.376
6.80	0.366	0.365	0.360	0.364
7.00	0.370	0.378	0.378	0.375
7.21	0.370	0.369	0.370	0.370
7.42	0.355	0.356	0.356	0.356
7.62	0.355	0.356	0.356	0.356
7.82	0.355	0.355	0.355	0.355
8.07	0.341	0.342	0.340	0.341
8.32	0.343	0.343	0.343	0.343
8.39	0.331	0.331	0.331	0.331
8.59	0.327	0.330	0.326	0.328
8.73	0.317	0.318	0.317	0.317
8.91	0.320	0.315	0.321	0.319
9.09	0.298	0.298	0.294	0.297
9.26	0.295	0.295	0.295	0.295
9.30	0.295	0.292	0.294	0.294
9.54	0.297	0.297	0.297	0.297
9.69	0.304	0.304	0.306	0.305
9.73	0.295	0.295	0.296	0.295
9.86	0.285	0.284	0.288	0.286
10.23	0.284	0.284	0.284	0.284
10.35	0.281	0.284	0.282	0.282
10.40	0.287	0.287	0.285	0.286
10.57	0.279	0.279	0.279	0.279
10.68	0.283	0.285	0.285	0.284
10.95	0.285	0.285	0.286	0.285
11.13	0.283	0.287	0.284	0.285
11.14	0.258	0.269	0.268	0.265
11.16	0.279	0.279	0.280	0.279
11.53	0.290	0.291	0.290	0.290

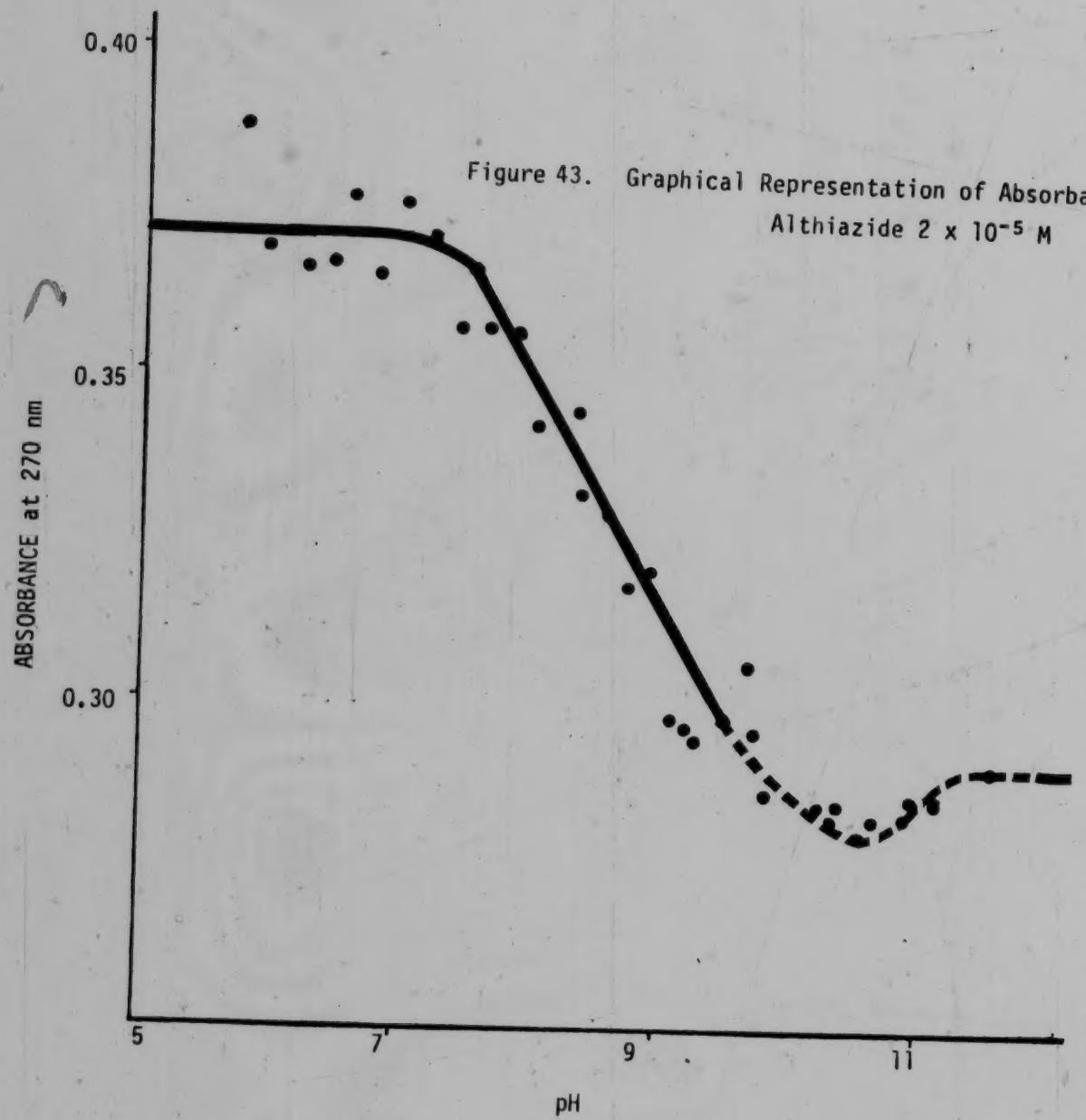


Figure 43. Graphical Representation of Absorbance-pH Data of Althiazide 2 x 10^{-5} M

Table 15. Data for the Determination of the Overlapping pKa Values of Flumethiazide

Flumethiazide 2 x 10^{-5} M
Analytical Wavelength: 270 nm
Pathlength: 1 cm
Ionic Strength: 0.01 M
Temperature: 24 ± 1°C

	Absorbance			
pH	#1	#2	#3	Average
4.15	0.160	0.159	0.157	0.159
4.33	0.160	0.159	0.159	0.159
4.50	0.159	0.159	0.154	0.157
4.73	0.157	0.158	0.156	0.157
4.88	0.154	0.155	0.154	0.154
5.05	0.151	0.151	0.149	0.150
5.21	0.147	0.146	0.149	0.147
5.56	0.142	0.142	0.139	0.141
5.75	0.139	0.139	0.133	0.137
5.93	0.106	0.102	0.100	0.103
6.12	0.082	0.081	0.079	0.081
6.30	0.071	0.071	0.072	0.071
6.46	0.070	0.069	0.071	0.070
6.61	0.068	0.069	0.069	0.069
6.80	0.065	0.065	0.065	0.065
6.99	0.051	0.051	0.050	0.051
7.26	0.049	0.050	0.049	0.049
7.55	0.051	0.048	0.049	0.049
7.64	0.042	0.042	0.041	0.042
7.80	0.033	0.034	0.035	0.034
7.98	0.033	0.030	0.031	0.031
8.22	0.029	0.030	0.031	0.030
8.45	0.032	0.033	0.031	0.032
8.68	0.036	0.036	0.036	0.036
8.86	0.046	0.047	0.046	0.046
9.08	0.050	0.050	0.049	0.050
9.29	0.051	0.051	0.051	0.051
9.55	0.057	0.057	0.057	0.057
9.79	0.059	0.057	0.056	0.057
10.01	0.060	0.059	0.059	0.059
10.10	0.067	0.066	0.065	0.066
10.50	0.068	0.068	0.069	0.068
10.76	0.066	0.067	0.067	0.067

Figure 44. Graphical Representation of Absorbance-pH Data of Flumethiazide 2×10^{-5} M

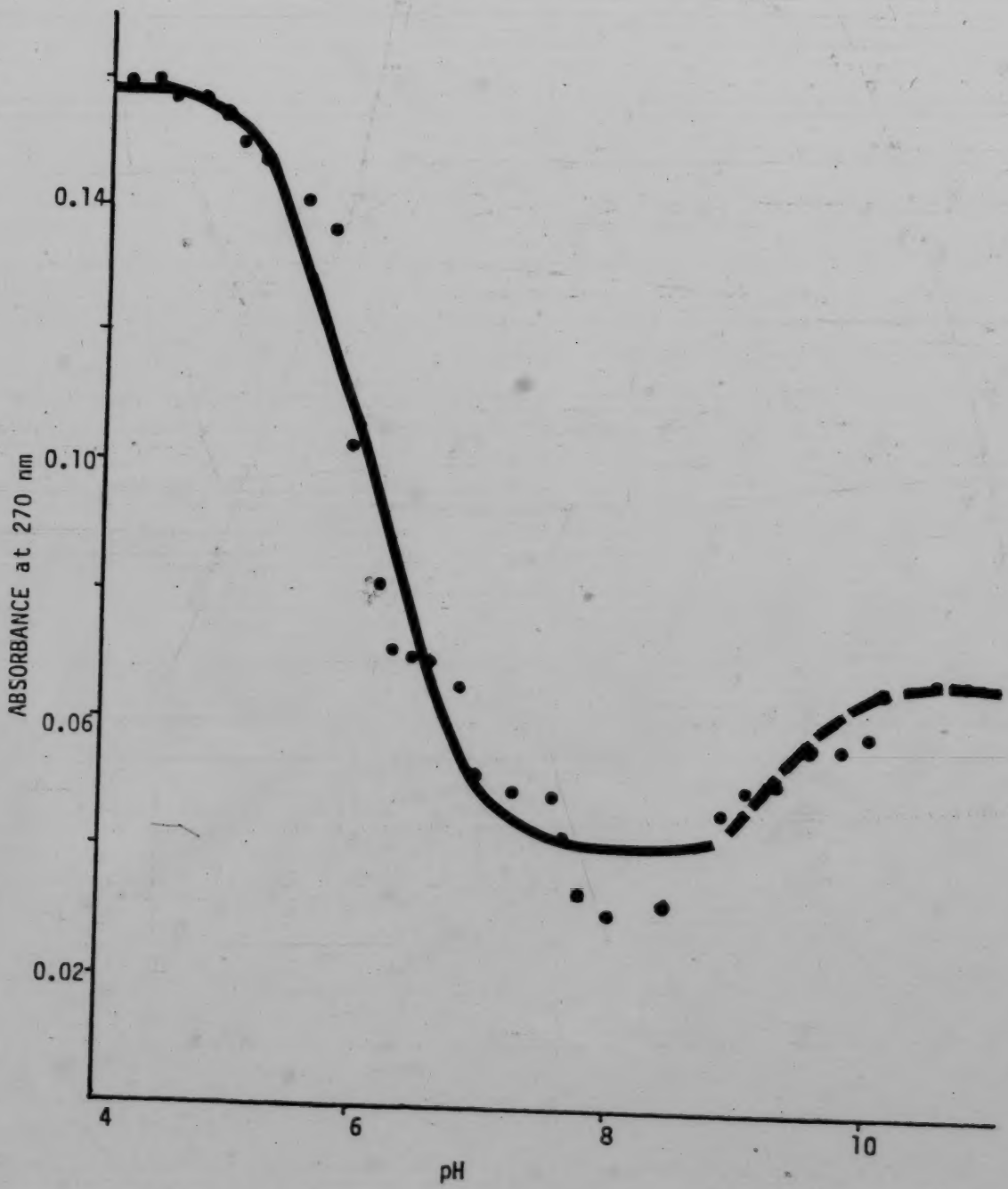


Table 16. Data for the Determination of the Overlapping pKa Values of Chlorothiazide

Chlorothiazide 2 x 10^{-5} M
Analytical Wavelength: 290 nm
Pathlength: 1 cm
Ionic Strength: 0.01 M
Temperature: 24 ± 1°C

	Absorbance			
pH	#1	#2	#3	Average
4.17	0.090	0.090	0.091	0.090
4.37	0.111	0.108	0.108	0.109
4.55	0.113	0.112	0.109	0.111
4.76	0.104	0.103	0.103	0.103
4.92	0.102	0.103	0.102	0.102
5.25	0.094	0.094	0.093	0.094
5.42	0.102	0.103	0.102	0.102
5.57	0.108	0.109	0.109	0.109
5.94	0.114	0.114	0.114	0.114
6.00	0.116	0.115	0.114	0.115
6.23	0.120	0.119	0.120	0.120
6.42	0.123	0.122	0.125	0.123
6.68	0.123	0.125	0.126	0.125
6.86	0.130	0.131	0.130	0.130
7.04	0.124	0.125	0.126	0.125
7.25	0.129	0.128	0.129	0.129
7.42	0.144	0.144	0.144	0.144
7.57	0.147	0.147	0.150	0.148
7.69	0.150	0.150	0.150	0.150
7.72	0.149	0.149	0.151	0.150
8.05	0.149	0.150	0.149	0.149
8.25	0.155	0.154	0.155	0.155
8.45	0.175	0.176	0.176	0.176
8.69	0.183	0.183	0.183	0.183
8.90	0.187	0.187	0.185	0.186
9.11	0.190	0.189	0.189	0.189
9.31	0.200	0.199	0.200	0.200
9.52	0.207	0.206	0.205	0.206
9.79	0.254	0.254	0.255	0.254
9.99	0.305	0.306	0.305	0.305
10.10	0.316	0.317	0.315	0.316
10.45	0.333	0.332	0.333	0.333
10.65	0.337	0.338	0.334	0.336
13.57	0.339	0.338	0.340	0.339

Figure 45. Graphical Representation of Absorbance pH-Data of Chlorothiazide 2 x 10^{-5} M

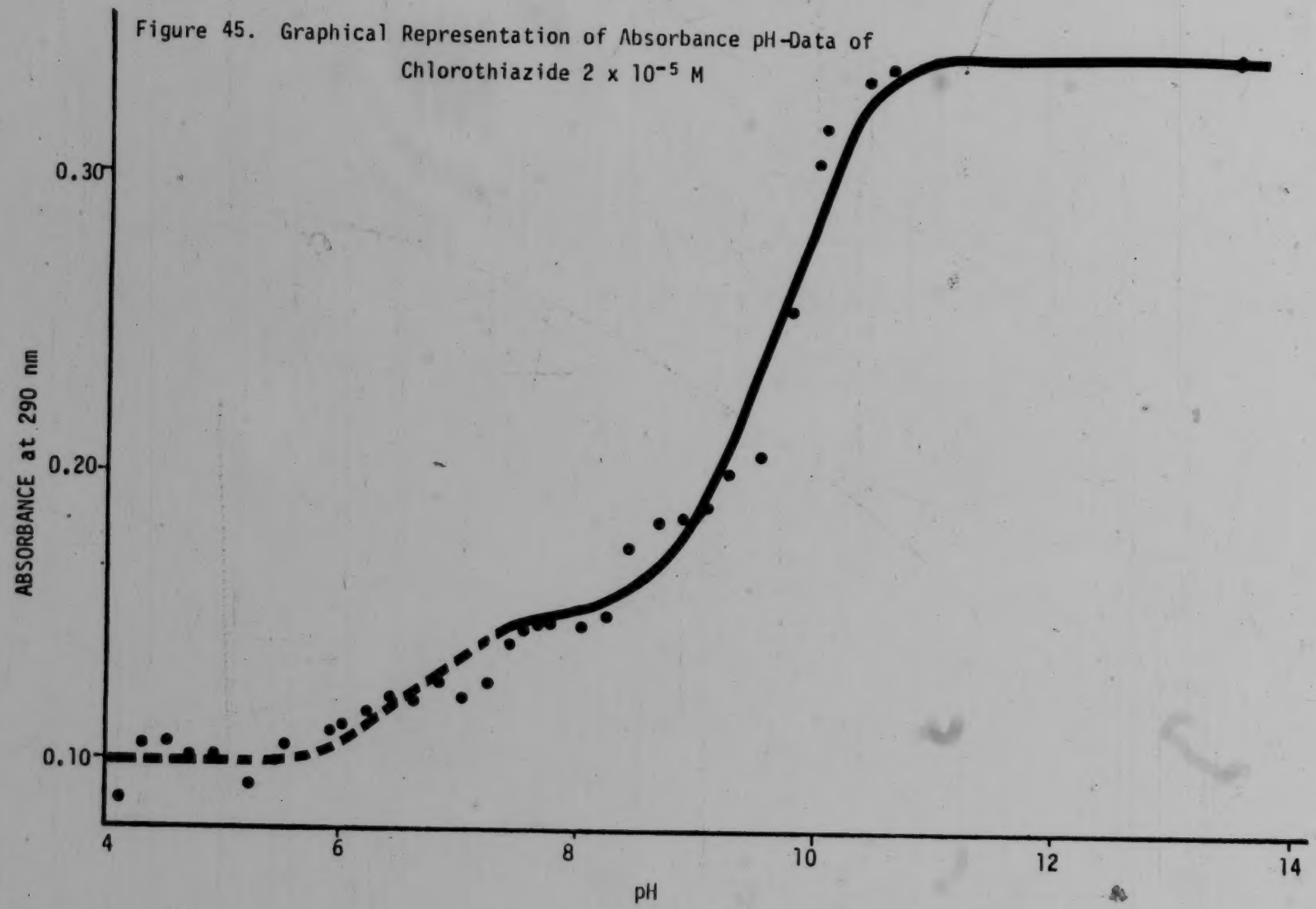


Table 17. Data for the Determination of the Overlapping pKa Values of Bendroflumethiazide

Bendroflumethiazide 2 x 10^{-5} M
Analytical Wavelength: 270 nm
Pathlength: 1 cm
Ionic Strength: 0.10 M
Temperature: 23.5 ± 1°C

	Absorbance			
pH	#1	#2	#3	Average
5.32	0.390	0.388	0.389	0.389
5.55	0.377	0.377	0.378	0.377
5.74	0.385	0.382	0.379	0.382
5.94	0.411	0.408	0.406	0.408
6.14	0.396	0.394	0.393	0.394
6.35	0.300	0.296	0.297	0.298
6.55	0.419	0.408	0.406	0.411
6.74	0.407	0.403	0.403	0.404
6.93	0.405	0.401	0.402	0.403
7.13	0.384	0.380	0.383	0.382
7.35	0.415	0.412	0.414	0.414
7.56	0.426	0.417	0.412	0.418
7.77	0.427	0.422	0.422	0.424
7.99	0.415	0.412	0.413	0.413
8.18	0.416	0.416	0.415	0.415
8.40	0.403	0.401	0.402	0.402
8.57	0.395	0.393	0.394	0.394
8.76	0.390	0.384	0.387	0.387
8.91	0.370	0.373	0.370	0.371
9.09	0.361	0.360	0.362	0.361
9.28	0.353	0.353	0.353	0.353
9.47	0.337	0.336	0.336	0.336
9.67	0.335	0.333	0.333	0.334
9.84	0.331	0.331	0.331	0.331
10.06	0.337	0.337	0.337	0.337
10.25	0.332	0.330	0.332	0.331
10.40	0.346	0.346	0.347	0.346
10.53	0.345	0.344	0.346	0.345
10.69	0.346	0.346	0.346	0.346
13.78	0.374	0.374	0.374	0.374

Figure 46. Graphical Representation of Absorbance-pH Data of Bendroflumethiazide 2 x 10^{-5} M

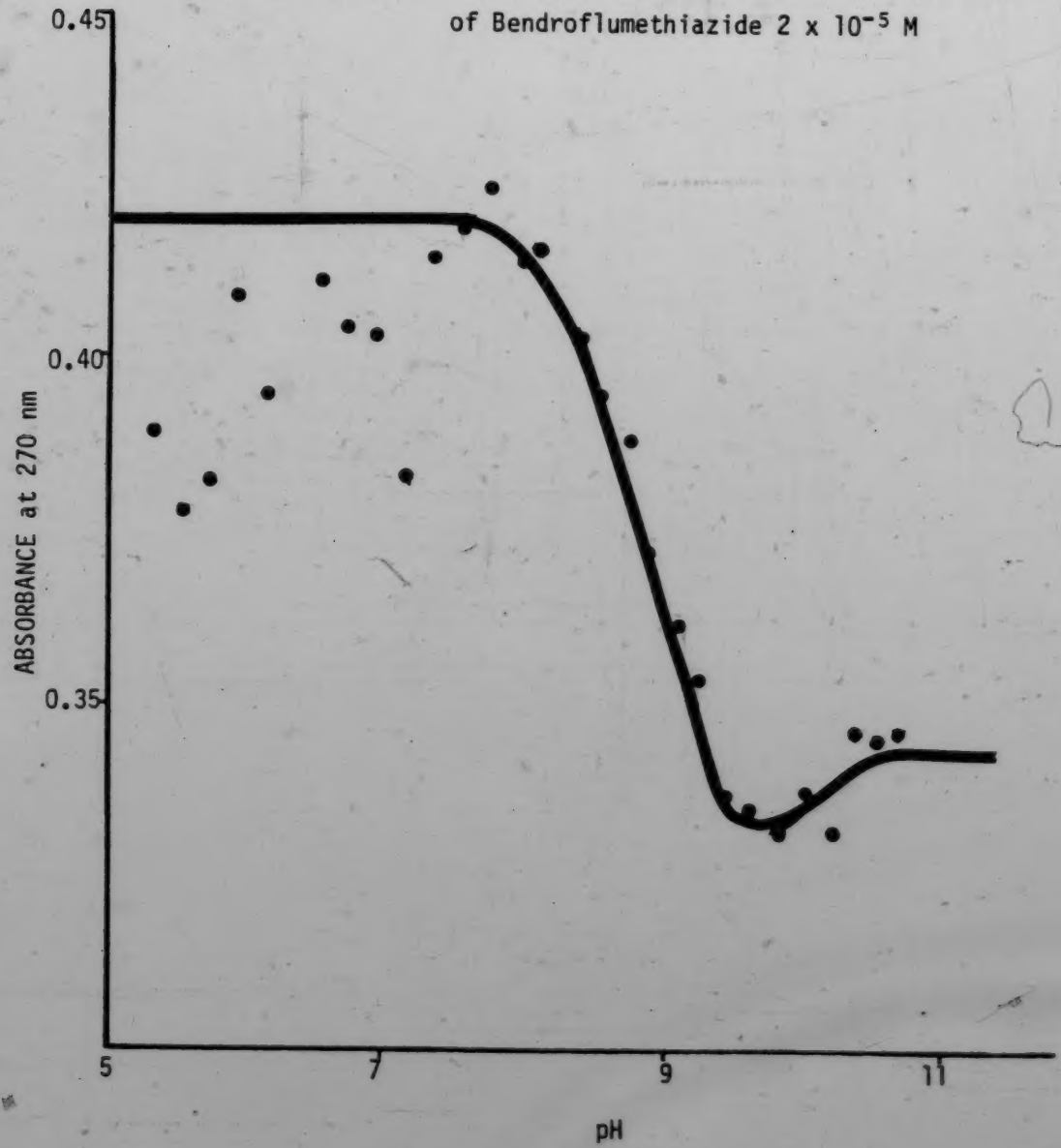


Table 18. Data for the Determination of the Overlapping pKa Values of Cyclothiazide

Cyclothiazide 2×10^{-5} M
Analytical Wavelength: 270 nm
Pathlength: 1 cm
Ionic Strength: 0.10 M
Temperature: 24 ± 1°C

	Absorbance			
pH	#1	#2	#3	Average
7.07	0.268	0.269	0.269	0.269
7.18	0.265	0.265	0.263	0.264
7.37	0.269	0.269	0.271	0.270
7.58	0.269	0.268	0.268	0.268
7.78	0.271	0.273	0.271	0.272
8.05	0.271	0.271	0.270	0.271
8.22	0.263	0.265	0.266	0.265
8.46	0.268	0.269	0.270	0.269
8.61	0.270	0.271	0.269	0.270
8.75	0.265	0.267	0.268	0.267
8.95	0.274	0.273	0.273	0.273
9.10	0.267	0.267	0.266	0.267
9.25	0.265	0.264	0.266	0.265
9.30	0.258	0.257	0.257	0.257
9.40	0.258	0.259	0.260	0.259
9.67	0.248	0.250	0.250	0.249
9.80	0.244	0.244	0.244	0.244
9.95	0.236	0.236	0.236	0.236
10.10	0.228	0.229	0.230	0.229
10.25	0.224	0.223	0.222	0.223
10.35	0.219	0.218	0.219	0.219
10.45	0.219	0.217	0.216	0.217
10.55	0.217	0.217	0.218	0.217
10.65	0.219	0.220	0.221	0.220
10.75	0.225	0.224	0.225	0.225
10.85	0.225	0.224	0.224	0.224
10.95	0.230	0.230	0.230	0.230
11.13	0.229	0.229	0.231	0.230
11.32	0.229	0.230	0.231	0.230

Figure 47. Graphical Representation of Absorbance pH-Data of Cyclothiazide 2 x 10^{-5} M

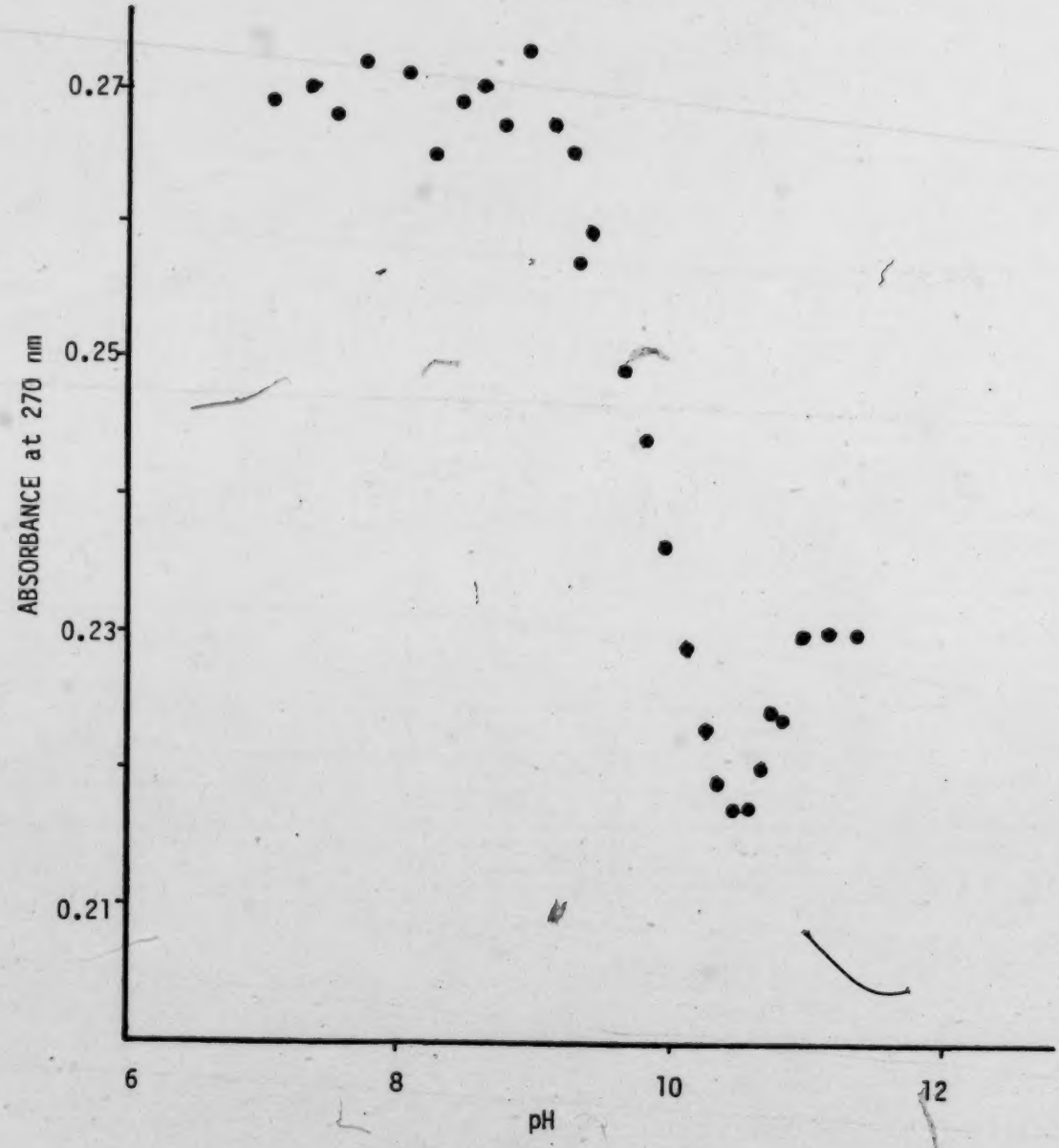


Table 19. Data for the Determination of the Overlapping pKa Values of Cyclopenthiazide

Cyclopenthiazide 2×10^{-5} M
Analytical Wavelength: 270 nm
Pathlength: 1 cm
Ionic Strength: 0.10 M
Temperature: 22 ± 1°C

	Absorbance			
pH	#1	#2	#3	Average
5.81	0.362	0.361	0.361	0.361
6.00	0.357	0.357	0.358	0.357
6.20	0.344	0.345	0.345	0.345
6.37	0.359	0.360	0.359	0.359
6.61	0.349	0.349	0.349	0.349
6.79	0.350	0.349	0.349	0.349
6.98	0.343	0.343	0.344	0.343
7.19	0.360	0.360	0.360	0.360
7.41	0.349	0.350	0.348	0.349
7.62	0.349	0.346	0.346	0.347
7.83	0.351	0.351	0.351	0.351
8.08	0.350	0.346	0.348	0.348
8.35	0.349	0.348	0.348	0.348
8.38	0.349	0.350	0.353	0.351
8.59	0.347	0.346	0.347	0.347
8.73	0.336	0.334	0.335	0.335
8.91	0.336	0.333	0.336	0.335
9.10	0.295	0.296	0.299	0.297
9.29	0.315	0.306	0.307	0.309
9.39	0.292	0.294	0.298	0.295
9.58	0.293	0.292	0.292	0.292
9.76	0.291	0.290	0.292	0.291
9.84	0.292	0.292	0.292	0.292
9.88	0.290	0.291	0.290	0.290
10.30	0.282	0.283	0.282	0.282
10.50	0.274	0.274	0.274	0.274
10.50	0.278	0.278	0.278	0.278
10.71	0.280	0.281	0.280	0.280
10.75	0.290	0.290	0.290	0.290
10.99	0.279	0.278	0.279	0.279
11.21	0.284	0.288	0.285	0.286
11.56	0.284	0.284	0.284	0.284
11.66	0.284	0.283	0.282	0.283
11.69	0.283	0.282	0.284	0.283
12.04	0.297	0.295	0.303	0.298

Figure 48. Graphical Representation of Absorbance pH-Data of Cyclopenthiazide 2 x 10^{-5} M

COMPUTER INPUT AND

OUTPUT DATA

(Tables 20 - 29)

Table 20. Computer Input and Output Data for Hydrochlorothiazide

```
*CHANGE LINE 1.8 TO D 26 FOR 20 DEGREES C
*1.8 D 26
*GO


*** RESOLUTION OF OVERLAPPING PKA VALUES ***

INPUT -1 FOR DIBASIC ACIDS, 0 FOR AMPHOLYTES, 1 FOR DIACIDIC BASES:-1

ABSORBANCE OF DIPROTONATED SPECIES :.363
ABSORBANCE OF NON-PROTONATED SPECIES :.305
IONIC STRENGTH :.1
PATH LENGTH IN CM :1
CONCENTRATION IN MOLES PER LITRE :.00002
TOTAL NUMBER OF PAIRS OF DATA :9
NUMBER OF PAIRS OF DATA FOR THE STRONGER SET :5
INPUT DATA IN ORDER OF INCREASING PH

PH:8.94   H+= 0.114809E-08  ABS:.339  EPSILON= 0.169500E+05  OK?:0
PH:9.09   H+= 0.812809E-09  ABS:.329  EPSILON= 0.164500E+05  OK?:0
PH:9.29   H+= 0.512819E-09  ABS:.323  EPSILON= 0.161500E+05  OK?:0
PH:9.49   H+= 0.323579E-09  ABS:.300  EPSILON= 0.150000E+05  OK?:0
PH:9.67   H+= 0.213783E-09  ABS:.284  EPSILON= 0.142000E+05  OK?:0

PH:10.62  H+= 0.239854E-10  ABS:.270  EPSILON= 0.135000E+05  OK?:0
PH:10.77  H+= 0.169805E-10  ABS:.272  EPSILON= 0.136000E+05  OK?:0
PH:10.97  H+= 0.107142E-10  ABS:.281  EPSILON= 0.140500E+05  OK?:0
PH:11.31  H+= 0.489714E-11  ABS:.296  EPSILON= 0.148000E+05  OK?:0
```

Table 20 continued...

K1	K2	EPSILON M
= 0.191630E-09	= 0.412137E-10	= 0.110386E+05
= 0.223414E-09	= 0.570115E-10	= 0.116955E+05
= 0.209992E-09	= 0.509235E-10	= 0.114424E+05
= 0.217586E-09	= 0.544604E-10	= 0.115895E+05
= 0.213556E-09	= 0.526147E-10	= 0.115127E+05
= 0.215795E-09	= 0.536487E-10	= 0.115557E+05
= 0.214544E-09	= 0.530739E-10	= 0.115318E+05
= 0.215245E-09	= 0.533969E-10	= 0.115452E+05
= 0.214859E-09	= 0.532192E-10	= 0.115379E+05
= 0.215077E-09	= 0.533193E-10	= 0.115420E+05
= 0.214945E-09	= 0.532585E-10	= 0.115395E+05
= 0.215012E-09	= 0.532894E-10	= 0.115408E+05
= 0.214981E-09	= 0.532752E-10	= 0.115402E+05
= 0.215001E-09	= 0.532847E-10	= 0.115406E+05
= 0.215000E-09	= 0.532839E-10	= 0.115405E+05
= 0.330180E-09	= 0.878102E-10	= 0.129762E+05
= 0.811370E-09	= 0.126032E-09	= 0.145855E+05
= 0.435773E-09	= 0.103427E-09	= 0.136256E+05
= 0.599849E-09	= 0.116783E-09	= 0.141810E+05
= 0.490729E-09	= 0.108895E-09	= 0.138530E+05
= 0.549858E-09	= 0.113558E-09	= 0.140469E+05
= 0.513351E-09	= 0.110806E-09	= 0.139324E+05
= 0.534296E-09	= 0.112431E-09	= 0.140000E+05
= 0.521698E-09	= 0.111469E-09	= 0.139606E+05
= 0.529015E-09	= 0.112033E-09	= 0.139835E+05
= 0.524743E-09	= 0.111706E-09	= 0.139698E+05
= 0.527226E-09	= 0.111897E-09	= 0.139778E+05
= 0.525771E-09	= 0.111785E-09	= 0.139731E+05
= 0.526684E-09	= 0.111855E-09	= 0.139761E+05
= 0.526090E-09	= 0.111810E-09	= 0.139742E+05
= 0.526391E-09	= 0.111833E-09	= 0.139751E+05
= 0.526267E-09	= 0.111823E-09	= 0.139747E+05
= 0.526324E-09	= 0.111828E-09	= 0.139749E+05
= 0.526320E-09	= 0.111828E-09	= 0.139749E+05
= 0.305290E-09	= 0.825557E-10	= 0.127577E+05

Table 20 continued...

H+	X	Y	PKA
= 0.114809E-08	= -0.340396E-18	= -0.133185E-08	= 0.956465E+01
= 0.812809E-09	= -0.342408E-18	= -0.117647E-08	= 0.950441E+01
= 0.512819E-09	= -0.213805E-18	= -0.909269E-09	= 0.958731E+01
= 0.323579E-09	= -0.482643E-18	= -0.136522E-08	= 0.947704E+01
= 0.213783E-09	= 0.629003E-19	= 0.138138E-09	= 0.954511E+01
= 0.239854E-10	= 0.559252E-21	= 0.478585E-11	= 0.115296E+02
= 0.169805E-10	= 0.290891E-21	= 0.407766E-11	= 0.115052E+02
= 0.107142E-10	= 0.143490E-21	= 0.542769E-11	= 0.113047E+02
= 0.489714E-11	= 0.653158E-22	= 0.104550E-10	= 0.109896E+02

AVERAGE PKA1= 0.953571E+01
AVERAGE PKA2= 0.113323E+02

Table 21. Computer Input and Output Data for Hydroflumethiazide

```
*CHANGE LINE 1.8 TO D 26 FOR 20 DEGREES C
*C HYDROFLUMETHIAZIDE
*GO


*** RESOLUTION OF OVERLAPPING PKA VALUES ***

INPUT -1 FOR DIBASIC ACIDS, 0 FOR AMPHOLYTES, 1 FOR DIACIDIC BASES:-1


ABSORBANCE OF DIPROTONATED SPECIES :.353
ABSORBANCE OF NON-PROTONATED SPECIES :.320
IONIC STRENGTH :.1
PATH LENGTH IN CM :1
CONCENTRATION IN MOLES PER LITRE :.00002
TOTAL NUMBER OF PAIRS OF DATA :11
NUMBER OF PAIRS OF DATA FOR THE STRONGER SET :6
INPUT DATA IN ORDER OF INCREASING PH

PH:8.72   H+= 0.190536E-08  ABS:.330   EPSILON= 0.165000E+05  OK?:0
PH:9.09   H+= 0.812809E-09  ABS:.309   EPSILON= 0.154500E+05  OK?:0
PH:9.27   H+= 0.536995E-09  ABS:.306   EPSILON= 0.153000E+05  OK?:0
PH:9.47   H+= 0.338821E-09  ABS:.291   EPSILON= 0.145500E+05  OK?:0
PH:9.64   H+= 0.229072E-09  ABS:.287   EPSILON= 0.143500E+05  OK?:0
PH:9.78   H+= 0.165947E-09  ABS:.278   EPSILON= 0.139000E+05  OK?:0

PH:10.71  H+= 0.194961E-10  ABS:.272   EPSILON= 0.136000E+05  OK?:0
PH:10.92  H+= 0.120216E-10  ABS:.277   EPSILON= 0.138500E+05  OK?:0
PH:11.22  H+= 0.602493E-11  ABS:.292   EPSILON= 0.146000E+05  OK?:0
PH:11.36  H+= 0.436472E-11  ABS:.291   EPSILON= 0.145500E+05  OK?:0
PH:11.58  H+= 0.263031E-11  ABS:.292   EPSILON= 0.146000E+05  OK?:0
```

Table 21 continued...

K1	K2	EPSILON M
= 0.508652E-09	= 0.240805E-10	= 0.131015E+05
= 0.479396E-09	= 0.159331E-10	= 0.129051E+05
= 0.495007E-09	= 0.204005E-10	= 0.130128E+05
= 0.487140E-09	= 0.181850E-10	= 0.129594E+05
= 0.491256E-09	= 0.193530E-10	= 0.129875E+05
= 0.489136E-09	= 0.187538E-10	= 0.129731E+05
= 0.490241E-09	= 0.190666E-10	= 0.129806E+05
= 0.489672E-09	= 0.189057E-10	= 0.129767E+05
= 0.489964E-09	= 0.189884E-10	= 0.129787E+05
= 0.489813E-09	= 0.189458E-10	= 0.129777E+05
= 0.489895E-09	= 0.189689E-10	= 0.129783E+05
= 0.489847E-09	= 0.189555E-10	= 0.129779E+05
= 0.489870E-09	= 0.189618E-10	= 0.129781E+05
= 0.489855E-09	= 0.189574E-10	= 0.129780E+05
= 0.489873E-09	= 0.189630E-10	= 0.129781E+05
= 0.489860E-09	= 0.189590E-10	= 0.129780E+05
= 0.489866E-09	= 0.189607E-10	= 0.129781E+05
= 0.489864E-09	= 0.189600E-10	= 0.129780E+05
= 0.606128E-09	= 0.455504E-10	= 0.136190E+05
= 0.672252E-09	= 0.565702E-10	= 0.138846E+05
= 0.671056E-09	= 0.563902E-10	= 0.138803E+05
= 0.671102E-09	= 0.563970E-10	= 0.138804E+05
= 0.671093E-09	= 0.563957E-10	= 0.138804E+05
= 0.671101E-09	= 0.563970E-10	= 0.138804E+05
= 0.671097E-09	= 0.563963E-10	= 0.138804E+05
= 0.566334E-09	= 0.376782E-10	= 0.134292E+05

Table 21 continued...

H+	X	Y	PKA
= 0.190536E-08	=-0.305479E-17	=-0.550469E-08	= 0.925273E+01
= 0.812809E-09	= 0.966806E-18	= 0.140484E-08	= 0.917376E+01
= 0.536995E-09	= 0.354170E-18	= 0.675110E-09	= 0.930372E+01
= 0.338821E-09	= 0.897917E-19	= 0.123198E-09	= 0.925323E+01
= 0.229072E-09	= 0.383950E-19	= 0.601347E-10	= 0.940610E+01
= 0.165947E-09	= 0.179908E-19	= 0.175008E-10	= 0.948669E+01
= 0.194961E-10	= 0.234662E-21	= 0.652678E-12	= 0.126228E+02
= 0.120216E-10	= 0.934486E-22	= 0.110681E-11	= 0.120260E+02
= 0.602493E-11	= 0.289319E-22	= 0.237018E-11	= 0.116347E+02
= 0.436472E-11	= 0.149007E-22	= 0.158705E-11	= 0.118067E+02
= 0.263031E-11	= 0.551425E-23	= 0.103475E-11	= 0.119893E+02

AVERAGE PKA1= 0.931271E+01
AVERAGE PKA2= 0.120159E+02

Table 22. Computer Input and Output Data for Trichloromethiazide

```
*C TRICHLOROMETHIAZIDE
*GO


*** RESOLUTION OF OVERLAPPING PKA VALUES ***

INPUT -1 FOR DIBASIC ACIDS, 0 FOR AMPHOLYTES, 1 FOR DIACIDIC BASES:-1


ABSORBANCE OF DIPROTONATED SPECIES :.390
ABSORBANCE OF NON-PROTONATED SPECIES :.320
IONIC STRENGTH : .1
PATH LENGTH IN CM :1
CONCENTRATION IN MOLES PER LITRE :.00002
TOTAL NUMBER OF PAIRS OF DATA :9
NUMBER OF PAIRS OF DATA FOR THE STRONGER SET :5
INPUT DATA IN ORDER OF INCREASING PH

PH: 7.03   H+= 0.933260E-07  ABS:.360   EPSILON= 0.180000E+05  OK?:0
PH: 7.24   H+= 0.575426E-07  ABS:.355   EPSILON= 0.177500E+05  OK?:0
PH: 7.43   H+= 0.371517E-07  ABS:.340   EPSILON= 0.170000E+05  OK?:0
PH: 7.62   H+= 0.239877E-07  ABS:.334   EPSILON= 0.167000E+05  OK?:0
PH: 7.84   H+= 0.144541E-07  ABS:.315   EPSILON= 0.157500E+05  OK?:0

PH:10.70   H+= 0.199508E-10  ABS:.304   EPSILON= 0.152000E+05  OK?:0
PH:10.90   H+= 0.125882E-10  ABS:.309   EPSILON= 0.154500E+05  OK?:0
PH:10.93   H+= 0.117480E-10  ABS:.309   EPSILON= 0.154500E+05  OK?:0
PH:11.10   H+= 0.794266E-11  ABS:.314   EPSILON= 0.157000E+05  OK?:0
```

Table 22 continued...

K1	K2	EPSILON M
= 0.324360E-07	= 0.211736E-08	= 0.147875E+05
= 0.344017E-07	= 0.213915E-08	= 0.149302E+05
= 0.335477E-07	= 0.213001E-08	= 0.148703E+05
= 0.339054E-07	= 0.213389E-08	= 0.148957E+05
= 0.337539E-07	= 0.213224E-08	= 0.148850E+05
= 0.338174E-07	= 0.213293E-08	= 0.148895E+05
= 0.337910E-07	= 0.213265E-08	= 0.148876E+05
= 0.338033E-07	= 0.213279E-08	= 0.148885E+05
= 0.337981E-07	= 0.213274E-08	= 0.148881E+05
= 0.337991E-07	= 0.213275E-08	= 0.148882E+05
= 0.337997E-07	= 0.213275E-08	= 0.148883E+05
= 0.337996E-07	= 0.213276E-08	= 0.148882E+05
= 0.247971E-07	= 0.199981E-08	= 0.140183E+05
= 0.250235E-07	= 0.200431E-08	= 0.140478E+05
= 0.250247E-07	= 0.200435E-08	= 0.140480E+05
= 0.250247E-07	= 0.200435E-08	= 0.140480E+05
= 0.287576E-07	= 0.206854E-08	= 0.144681E+05

H+	X	Y	PKA
= 0.933260E-07	=-0.279496E-14	=-0.908049E-07	= 0.750171E+01
= 0.575426E-07	=-0.145337E-14	=-0.609950E-07	= 0.760791E+01
= 0.371517E-07	=-0.179279E-14	=-0.629327E-07	= 0.753081E+01
= 0.239877E-07	=-0.146487E-14	=-0.626830E-07	= 0.761676E+01
= 0.144541E-07	=-0.518061E-15	=-0.157774E-07	= 0.753714E+01
= 0.199508E-10	= 0.565881E-21	= 0.621691E-11	= 0.112076E+02
= 0.125882E-10	= 0.274596E-21	= 0.681031E-11	= 0.111674E+02
= 0.117480E-10	= 0.239162E-21	= 0.635573E-11	= 0.111974E+02
= 0.794266E-11	= 0.145309E-21	= 0.763733E-11	= 0.111173E+02

AVERAGE PKA1= 0.755887E+01
AVERAGE PKA2= 0.111725E+02

Table 23. Computer Input and Output Data for Althiazide

```
?00 AT  0.00
*L G PKA2
*C ALTHIAZIDE
*GO



*** RESOLUTION OF OVERLAPPING PKA VALUES ***

INPUT -1 FOR DIBASIC ACIDS,0 FOR AMPHOLYTES,1 FOR DIACIDIC BASES:-1


ABSORBANCE OF DIPROTONATED SPECIES :.370
ABSORBANCE OF NON-PROTONATED SPECIES :.290
IONIC STRENGTH :.1
PATH LENGTH IN CM :1
CONCENTRATION IN MOLES PER LITRE :.00002
TOTAL NUMBER OF PAIRS OF DATA :10
NUMBER OF PAIRS OF DATA FOR THE STRONGER SET :6
INPUT DATA IN ORDER OF INCREASING PH

PH:8.07   H+= 0.100000E-07  ABS:.341   EPSILON= 0.170500E+05  OK?:1 XX
PH:8.07   H+= 0.851138E-08  ABS:.341   EPSILON= 0.170500E+05  OK?:0
PH:8.32   H+= 0.478630E-08  ABS:.343   EPSILON= 0.171500E+05  OK?:0
PH:8.39   H+= 0.407380E-08  ABS:.339   EPSILON= 0.169500E+05  OK?:0
PH:8.59   H+= 0.257040E-08  ABS:.328   EPSILON= 0.164000E+05  OK?:0
PH:8.73   H+= 0.186209E-08  ABS:.317   EPSILON= 0.158500E+05  OK?:0
PH:8.91   H+= 0.123027E-08  ABS:.319   EPSILON= 0.159500E+05  OK?:0

PH:10.35   H+= 0.446684E-10  ABS:.282   EPSILON= 0.141000E+05  OK?:0
PH:10.48   H+= 0.398107E-10  ABS:.286   EPSILON= 0.143000E+05  OK?:0
PH:10.57   H+= 0.269153E-10  ABS:.279   EPSILON= 0.139500E+05  OK?:0
PH:10.68   H+= 0.208930E-10  ABS:.284   EPSILON= 0.142000E+05  OK?:0
```

Table 23 continued...

K1	K2	EPSILON M
= 0.728163E-08	= 0.984979E-10	= 0.157577E+05
= 0.839164E-08	= 0.727288E-10	= 0.159264E+05
= 0.792108E-08	= 0.827711E-10	= 0.158607E+05
= 0.808568E-08	= 0.791254E-10	= 0.158845E+05
= 0.802333E-08	= 0.804889E-10	= 0.158756E+05
= 0.804629E-08	= 0.799842E-10	= 0.158789E+05
= 0.803774E-08	= 0.801717E-10	= 0.158777E+05
= 0.804091E-08	= 0.801022E-10	= 0.158781E+05
= 0.803974E-08	= 0.801280E-10	= 0.158780E+05
= 0.804017E-08	= 0.801184E-10	= 0.158780E+05
= 0.804001E-08	= 0.801220E-10	= 0.158780E+05
= 0.804007E-08	= 0.801206E-10	= 0.158780E+05
= 0.347387E-08	= 0.312037E-09	= 0.143599E+05
= 0.372752E-08	= 0.284250E-09	= 0.145418E+05
= 0.370707E-08	= 0.286349E-09	= 0.145281E+05
= 0.370861E-08	= 0.286190E-09	= 0.145291E+05
= 0.370849E-08	= 0.286202E-09	= 0.145290E+05
= 0.370850E-08	= 0.286201E-09	= 0.145290E+05
= 0.507579E-08	= 0.183161E-09	= 0.152035E+05

H+	X	Y	PKA
= 0.851138E-08	=-0.149816E-16	=-0.288636E-08	= 0.825632E+01
= 0.478630E-08	=-0.424441E-17	=-0.164646E-08	= 0.853751E+01
= 0.407380E-08	=-0.381852E-17	=-0.136001E-08	= 0.848883E+01
= 0.257040E-08	=-0.265580E-17	=-0.758048E-09	= 0.833539E+01
= 0.186209E-08	=-0.247538E-17	=-0.417604E-09	= 0.797640E+01
= 0.123027E-08	=-0.968359E-18	=-0.296616E-09	= 0.806892E+01

076114

AVERAGE pKa_1 = 0.827723E+01

Table 24. Computer Input and Output Data for Flumethiazide

```
C FLUMETHIAZIDE
*** RESOLUTION OF OVERLAPPING PKA VALUES ***

INPUT -1 FOR DIBASIC ACIDS, 0 FOR AMPHOLYTES, 1 FOR DIACIDIC
                                                      BASES:-1

ABSORBANCE OF DIPROTONATED SPECIES :.159
ABSORBANCE OF NON-PROTONATED SPECIES:.067
IONIC STRENGTH :.01
PATH LENGTH IN CM :1
CONCENTRATION IN MOLES PER LITRE :.00002
TOTAL NUMBER OF PAIRS OF DATA :10
NUMBER OF PAIRS OF DATA FOR THE STRONGER SET :6
INPUT DATA IN ORDER OF INCREASING PH

PH: 5.93   H+= 0.117485E-05  ABS:.103   EPSILON= 0.515000E+04
PH: 6.12   H+= 0.758545E-06  ABS:.081   EPSILON= 0.405000E+04
PH: 6.30   H+= 0.501182E-06  ABS:.071   EPSILON= 0.355000E+04
PH: 6.46   H+= 0.346730E-06  ABS:.070   EPSILON= 0.350000E+04
PH: 6.61   H+= 0.245457E-06  ABS:.069   EPSILON= 0.345000E+04
PH: 6.80   H+= 0.158480E-06  ABS:.065   EPSILON= 0.325000E+04

PH: 8.68   H+= 0.208918E-08  ABS:.036   EPSILON= 0.180000E+04
PH: 8.86   H+= 0.138030E-08  ABS:.046   EPSILON= 0.230000E+04
PH: 9.08   H+= 0.831703E-09  ABS:.050   EPSILON= 0.250000E+04
PH: 9.29   H+= 0.512819E-09  ABS:.051   EPSILON= 0.255000E+04

      K1              K2            EPSILON M
= 0.180400E-05  = 0.242118E-07  = 0.265824E+04
= 0.176634E-05  = 0.218631E-07  = 0.263958E+04
= 0.177174E-05  = 0.222062E-07  = 0.264230E+04
= 0.177097E-05  = 0.221573E-07  = 0.264191E+04
= 0.177108E-05  = 0.221640E-07  = 0.264197E+04
= 0.177107E-05  = 0.221634E-07  = 0.264196E+04

= 0.836121E-06  = 0.103280E-06  = 0.164484E+04
= 0.838683E-06  = 0.102554E-06  = 0.165061E+04
= 0.838672E-06  = 0.102557E-06  = 0.165059E+04
= 0.838672E-06  = 0.102557E-06  = 0.165059E+04

= 0.113831E-05  = 0.401974E-07  = 0.214628E+04

      H+              X               Y              PKA
= 0.117485E-05  =-0.142482E-11  =-0.144146E-05  = 0.601698E+01
= 0.758545E-06  =-0.212736E-11  =-0.151678E-05  = 0.586443E+01
= 0.501182E-06  =-0.366713E-11  =-0.258633E-05  = 0.585504E+01
= 0.346730E-06  =-0.236681E-11  =-0.230073E-05  = 0.599521E+01
= 0.245457E-06  =-0.179919E-11  =-0.235286E-05  = 0.612386E+01
= 0.158480E-06  =-0.783350E-12  =-0.128608E-05  = 0.620156E+01

717 AT 15.10
*                                   AVERAGE pKa1  = 0.600950E+01
```

Table 25. Computer Input and Output Data for Chlorothiazide

```
 C CHLOROTHIAZIDE

*** RESOLUTION OF OVERLAPPING PKA VALUES ***

INPUT -1 FOR DIBASIC ACIDS, 0 FOR AMPHOLYTES, 1 FOR DIACIDIC
                                                      BASES:-1

ABSORBANCE OF DIPROTONATED SPECIES :.339
ABSORBANCE OF NON-PROTONATED SPECIES :.104
IONIC STRENGTH :.01
PATH LENGTH IN CM :1
CONCENTRATION IN MOLES PER LITRE :.00002
TOTAL NUMBER OF PAIRS OF DATA :8
NUMBER OF PAIRS OF DATA FOR THE STRONGER SET :5
INPUT DATA IN ORDER OF INCREASING PH

PH: 5.94   H+= 0.114816E-05  ABS:.114   EPSILON= 0.570000E+04
PH: 6.00   H+= 0.999985E-06  ABS:.115   EPSILON= 0.575000E+04
PH: 6.23   H+= 0.588817E-06  ABS:.120   EPSILON= 0.600000E+04
PH: 6.42   H+= 0.380183E-06  ABS:.123   EPSILON= 0.615000E+04
PH: 6.68   H+= 0.208926E-06  ABS:.125   EPSILON= 0.625000E+04

PH: 9.79   H+= 0.162170E-09  ABS:.254   EPSILON= 0.127000E+05
PH: 9.99   H+= 0.102326E-09  ABS:.305   EPSILON= 0.152500E+05
PH: 10.10  H+= 0.794287E-10  ABS:.316   EPSILON= 0.158000E+05

      K1              K2              EPSILON M
=-0.173887E-04  = 0.317696E-08  = 0.635827E+04
=-0.169880E-04  = 0.472848E-08  = 0.637414E+04
=-0.168109E-04  = 0.543782E-08  = 0.638139E+04
=-0.167313E-04  = 0.576161E-08  = 0.638471E+04
=-0.166950E-04  = 0.590990E-08  = 0.638622E+04
=-0.166789E-04  = 0.597611E-08  = 0.638690E+04
=-0.166714E-04  = 0.600686E-08  = 0.638722E+04
=-0.166679E-04  = 0.602148E-08  = 0.638737E+04
=-0.166663E-04  = 0.602786E-08  = 0.638743E+04
=-0.166656E-04  = 0.603080E-08  = 0.638746E+04
=-0.166654E-04  = 0.603181E-08  = 0.638747E+04
=-0.166651E-04  = 0.603307E-08  = 0.638748E+04
=-0.166652E-04  = 0.603248E-08  = 0.638748E+04

=-0.212329E-05  = 0.476103E-06  = 0.111959E+05
=-0.212375E-05  = 0.475987E-06  = 0.111947E+05
=-0.212375E-05  = 0.475987E-06  = 0.111947E+05

=-0.376739E-05  = 0.241009E-06  = 0.879108E+04

      H+              X               Y              PKA
?17 AT 15.10
```

Table 26. *Manual Calculations for Resolution of the Overlapping pKa Values of Chlorothiazide and Bendroflumethiazide

Chlorothiazide

H+	X	Y	pKa
= 0.162170E-09	=-0.373299E-19	=-0.621480E-10	= 0.979360E+01
= 0.102326E-09	=-0.117528E-20	=-0.483549E-10	= 0.968440E+01
= 0.794287E-10	=-0.454185E-21	=-0.386175E-10	= 0.958670E+01

average pKa_2 = 0.968823E+01

Bendroflumethiazide

Epsilon M

Epsilon M_1 = 0.157471E+05
Epsilon M_2 = 0.164099E+05

Average Epsilon M = 0.160794E+05

K_1	K_2	Epsilon M
= 0.121883E-08	=-0.292203E-09	= 0.160794E+05

H+	X	Y	pKa
= 0.269153E-08	=-0.142691E-17	=-0.190182E-08	= 0.90523E+01
= 0.173780E-08	=-0.883884E-18	=-0.129860E-08	= 0.90566E+01
= 0.123027E-08	=-0.107876E-17	=-0.113892E-08	= 0.88948E+01
= 0.812831E-09	=-0.944993E-18	=-0.100032E-08	= 0.88747E+01
= 0.524807E-09	=-0.958613E-18	=-0.110306E-08	= 0.89274E+01

H+	X	Y	pKa
= 0.144544E-09	= 0.450780E-19	= 0.424808E-10	= 0.106653E+02
= 0.870964E-10	= 0.254392E-19	= 0.698584E-10	= 0.103098E+02

average pKa_1 = 0.896116E+01
average pKa_2 = 0.104876E+02

*Computer input and output data for chlorothiazide and bendroflumethiazide were used in the manual calculations.

Table 27. Computer Input and Output Data for Bendroflumethiazide

```
*C BENDROFLUMETHIAZIDE
*** RESOLUTION OF OVERLAPPING PKA VALUES ***

INPUT -1 FOR DIBASIC ACIDS,0 FOR AMPHOLYTES,1 FOR DIACIDIC
                                                BASES:-1

ABSORBANCE OF DIPROTONATED SPECIES :.420
ABSORBANCE OF NON-PROTONATED SPECIES :.346
IONIC STRENGTH :.1
PATH LENGTH IN CM :1
CONCENTRATION IN MOLES PER LITRE :.00002
TOTAL NUMBER OF PAIRS OF DATA :7
NUMBER OF PAIRS OF DATA FOR THE STRONGER SET :5
INPUT DATA IN ORDER OF INCREASING PH

PH:8.57   H+= 0.269153E-08  ABS:.394   EPSILON= 0.197000E+05
PH:8.76   H+= 0.173780E-08  ABS:.387   EPSILON= 0.193500E+05
PH:8.91   H+= 0.123027E-08  ABS:.371   EPSILON= 0.185500E+05
PH:9.09   H+= 0.812831E-09  ABS:.361   EPSILON= 0.180500E+05
PH:9.28   H+= 0.524807E-09  ABS:.353   EPSILON= 0.176500E+05

PH:9.84   H+= 0.144544E-09  ABS:.331   EPSILON= 0.165500E+05
PH:10.06  H+= 0.870964E-10  ABS:.337   EPSILON= 0.168500E+05
      K1                    K2                  EPSILON M
= 0.644664E-09   = 0.469591E-10   = 0.154807E+05
= 0.750484E-09   = 0.333069E-10   = 0.159147E+05
= 0.684674E-09   = 0.413012E-10   = 0.156605E+05
= 0.718681E-09   = 0.369873E-10   = 0.157977E+05
= 0.698890E-09   = 0.394467E-10   = 0.157195E+05
= 0.709736E-09   = 0.380819E-10   = 0.157629E+05
= 0.703579E-09   = 0.388516E-10   = 0.157384E+05
= 0.707008E-09   = 0.384213E-10   = 0.157521E+05
= 0.705077E-09   = 0.386631E-10   = 0.157444E+05
= 0.706158E-09   = 0.385276E-10   = 0.157487E+05
= 0.705551E-09   = 0.386036E-10   = 0.157463E+05
= 0.705891E-09   = 0.385610E-10   = 0.157476E+05
= 0.705700E-09   = 0.385849E-10   = 0.157469E+05
= 0.705807E-09   = 0.385715E-10   = 0.157473E+05
= 0.705747E-09   = 0.385790E-10   = 0.157471E+05
= 0.705781E-09   = 0.385748E-10   = 0.157472E+05
= 0.705762E-09   = 0.385772E-10   = 0.157471E+05
= 0.705772E-09   = 0.385758E-10   = 0.157472E+05
= 0.705767E-09   = 0.385766E-10   = 0.157471E+05

=-0.289210E-10   = 0.220810E-08   = 0.871739E+05
= 0.249636E-09   = 0.200173E-09   = 0.136096E+05
=-0.586304E-09   = 0.156379E-09   = 0.219452E+05
= 0.923424E-09   = 0.177305E-10   = 0.164099E+05
=-0.420408E-10   = 0.153457E-08   = 0.657609E+05*C

= 0.319970E-09   = 0.145212E-09   = 0.123570E+05*C
*C
```

Table 28. Computer Input and Output Data for Cyclothiazide

```
*C CYCLOTHIAZIDE
*GO


*** RESOLUTION OF OVERLAPPING PKA VALUES ***

INPUT -1 FOR DIBASIC ACIDS, 0 FOR AMPHOLYTES, 1 FOR DIACIDIC
                                                      BASES:-1

ABSORBANCE OF DIPROTONATED SPECIES :.269
ABSORBANCE OF NON-PROTONATED SPECIES :.230
IONIC STRENGTH :.1
PATH LENGTH IN CM :1
CONCENTRATION IN MOLES PER LITRE :.00002
TOTAL NUMBER OF PAIRS OF DATA :16
NUMBER OF PAIRS OF DATA FOR THE STRONGER SET :7
INPUT DATA IN ORDER OF INCREASING PH

PH: 9.30   H+= 0.501162E-09   ABS:.257   EPSILON= 0.128500E+05
PH: 9.40   H+= 0.398094E-09   ABS:.259   EPSILON= 0.129500E+05
PH: 9.67   H+= 0.213783E-09   ABS:.250   EPSILON= 0.125000E+05
PH: 9.80   H+= 0.158476E-09   ABS:.244   EPSILON= 0.122000E+05
PH: 9.95   H+= 0.112195E-09   ABS:.236   EPSILON= 0.118000E+05
PH:10.10   H+= 0.794287E-10   ABS:.229   EPSILON= 0.114500E+05
PH:10.25   H+= 0.562293E-10   ABS:.223   EPSILON= 0.111500E+05

PH:10.65   H+= 0.223853E-10   ABS:.220   EPSILON= 0.110000E+05
PH:10.75   H+= 0.177809E-10   ABS:.225   EPSILON= 0.112500E+05
PH:10.85   H+= 0.141244E-10   ABS:.224   EPSILON= 0.112000E+05
        K1                    K2                 EPSILON M
 = 0.874543E-10   = 0.259204E-10   = 0.101064E+05
 =-0.103713E-09   = 0.119639E-09   = 0.129421E+05
 =-0.509666E-10   = 0.164010E-09   = 0.142847E+05
 =-0.280736E-10   = 0.235155E-09   = 0.164374E+05
 =-0.171524E-10   = 0.336004E-09   = 0.194889E+05
 =-0.102590E-10   = 0.510198E-09   = 0.247597E+05
 =-0.634653E-11   = 0.777396E-09   = 0.328446E+05
 =-0.407084E-11   = 0.116907E-08   = 0.446957E+05
 =-0.266047E-11   = 0.174811E-08   = 0.622166E+05
 =-0.175290E-11   = 0.261346E-08   = 0.884004E+05
 =-0.115875E-11   = 0.391415E-08   = 0.127757E+06
 =-0.766974E-12   = 0.587433E-08   = 0.187068E+06
 =-0.507889E-12   = 0.8831
?00 AT  3.30
*GO
```

Table 29. Computer Input and Output Data for Cyclopenthiazide

```
*CHANGE LINE 1.8 TO D 26 FOR 20 DEGREES C
*1.8 D 26
*C CYCLOPENTHIAZIDE
*GO


*** RESOLUTION OF OVERLAPPING PKA VALUES ***

INPUT -1 FOR DIBASIC ACIDS, 0 FOR AMPHOLYTES, 1 FOR DIACIDIC
                                                    BASES:-1

ABSORBANCE OF DIPROTONATED SPECIES :.349
ABSORBANCE OF NON-PROTONATED SPECIES :.290
IONIC STRENGTH :.1
PATH LENGTH IN CM :1
CONCENTRATION IN MOLES PER LITRE :.00002
TOTAL NUMBER OF PAIRS OF DATA :7
NUMBER OF PAIRS OF DATA FOR THE STRONGER SET :5
INPUT DATA IN ORDER OF INCREASING PH

PH: 8.73   H+= 0.186197E-08  ABS:.335   EPSILON= 0.167500E+05
PH: 8.91   H+= 0.123017E-08  ABS:.335   EPSILON= 0.167500E+05
PH: 9.29   H+= 0.512819E-09  ABS:.309   EPSILON= 0.154500E+05
PH: 9.39   H+= 0.407363E-09  ABS:.295   EPSILON= 0.147500E+05
PH: 9.58   H+= 0.263016E-09  ABS:.292   EPSILON= 0.146000E+05

PH: 10.99  H+= 0.102320E-10  ABS:.279   EPSILON= 0.139500E+05
PH: 11.21  H+= 0.616549E-11  ABS:.286   EPSILON= 0.143000E+05

      K1                K2              EPSILON M
= 0.780286E-09   = 0.206232E-09   = 0.137451E+05
=-0.987706E-10   = 0.503772E-09   = 0.215182E+05
=-0.537612E-10   = 0.724885E-09   = 0.272947E+05
=-0.304315E-10   = 0.109687E-08   = 0.370127E+05
=-0.181742E-10   = 0.167501E-08   = 0.521163E+05
=-0.112636E-10   = 0.255564E-08   = 0.751225E+05
=-0.71
?00 AT  3.30
*
```

*Table 30. Acidity Constants of Various Benzothiadiazines and Diazoxide as Determined by Ultraviolet Spectrophotometry

Compound	pKa_1	pKa_2
hydrochlorothiazide	9.5 ± 0.1	11.3 ± 0.3
hydroflumethiazide	9.3 ± 0.2	12.0 ± 0.6
trichloromethiazide	7.6 ± 0.1	11.2 ± 0.1
althiazide	8.3 ± 0.3	-
bendroflumethiazide	9.0 ± 0.1	-
flumethiazide	6.0 ± 0.2	-
chlorothiazide	-	9.7 ± 0.1
methyclothiazide	9.5 ± 0.2	-
polythiazide	9.1 ± 0.1	-
diazoxide	8.4 ± 0.1	-

* The error estimates were obtained using the method of determining scatter by Albert and Serjeant (20).

Table 31. Comparisons of the Acidity Constants of Benzothiadiazines and Diazoxide Obtained by Various Techniques

Benzothiadiazine	Method of Determination: Aqueous Potentiometric Titration		Semiaqueous Potentiometric Titration		Ultraviolet* Spectrophotometry		Solubility Variation with pH	
	pKa_1	pKa_2	pKa_1	pKa_2	pKa_1	pKa_2	pKa_1	pKa_2
chlorothiazide	6.7	9.5 (11)	6.9	12.1 (7)	-	9.7		
	6.8	9.4 (7)						
	6.83	- (19)						
flumethiazide	6.44	- (19)	6.3	- (80)	6.0	-		
hydrochlorothiazide	7.0	9.2 (77)	8.7	- (80)	8.7	- (13)		
	7.9	9.2 (76)			9.5	11.3		
	8.6	9.9 (13)						
	8.80	- (19)						
hydroflumethiazide	8.9	10.7 (77)	8.5	- (80)	9.3	12.0		
	8.45	- (19)						
cyclothiazide			9.1	10.5 (78)				
			8.8	- (80)				
trichloromethiazide			6.9	- (80)	7.6	11.2		
bendroflumethiazide					9.0	-	8.53 - (18)	
methyclothiazide			9.4	- (79)	9.5			
			9.5	- (80)				
polythiazide			9.05	- (80)	9.12			
diazoxide					8.42			

*Data from Table 30.

Nuclear Magnetic Resonance in Determining the Order of Deprotonation

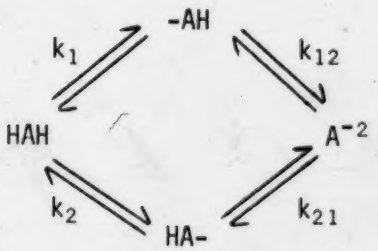
Benzothiadiazines are not amenable to pKa determination by conventional NMR spectrometry because their limited water solubility does not permit concentrations of 0.1 M in purely aqueous media. Aqueous solutions of 2×10^{-5} M can be achieved but this concentration can be increased by the addition of organic solvents, although only relative or comparative pKa values will then be determined.

NMR spectrometry is especially useful in deciding the order of protonation or deprotonation when overlapping ionizations occur, therefore, this method should be particularly valuable in determining the order of dissociation of the two acidic hydrogens of the thiazides and of the hydrothiazides. Theoretically, if more than one ionizing group is present the shift of one representative group is expected to begin before the shift of the other representative group begins, but this is not necessarily valid when the ionizations strongly overlap because simultaneous shifting will occur (59).

The acid dissociation constants of thiazides and of hydrothiazides can be described by the following deprotonations:

$$H_2A \overset{K_1}{\rightleftharpoons} H^+ + HA^- \overset{K_2}{\rightleftharpoons} H^+ + A^{-2},$$

where K_1 and K_2 represent the macroscopic acidity constants. A more detailed account of the dissociations involves the individual groups, as described by Rabenstein (59):



where k represents a microscopic dissociation constant, the last number in the subscript designates the group involved in the particular ionization step, and the first number in the subscript designates the group from which a proton has already ionized.

The ionization schemes for the macroscopic constants and the microscopic constants can be expressed in the following terms:

$$K_1 = \frac{[H^+]([HA^-]+[-AH])}{[HAH]} \quad \text{and} \quad K_2 = \frac{[H^+][A^{-2}]}{[HA^-]+[-AH]}$$

whereas $k_1 = \frac{[H^+][^-AH]}{[HAH]}$, $k_2 = \frac{[H^+][HA^-]}{[HAH]}$,

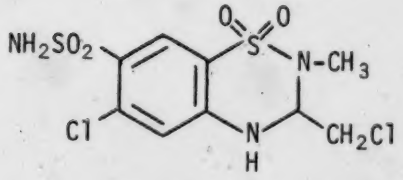
$$k_{12} = \frac{[H^+][A^{-2}]}{[-AH]}, \quad \text{and} \quad k_{21} = \frac{[H^+][A^{-2}]}{[HA^-]} .$$

Therefore, the relationship between the macroscopic constants and the microscopic constants is $K_1 = k_1 + k_2$ and $K_2 = \frac{k_{12} \cdot k_{21}}{k_{12} + k_{21}}$ and it is evident that the macroscopic constants are a composite of the microscopic constants.

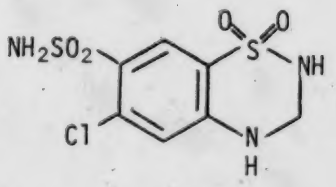
The chemical shift of the group adjacent to the ionizing group may depend on the protonation or deprotonation of only one of the functional groups (a unique resonance) or on the ionization of both groups (a common resonance)(58). Ideally, if a unique resonance could be located,

two titration curves would be evident and one would begin before the other, providing evidence of the order of ionization. The order of ionization is not directly evident when a common resonance is involved.

To determine the order of deprotonation, the representative benzothiadiazines methyclothiazide and hydrochlorothiazide were chosen as the model compounds.



methyclothiazide



hydrochlorothiazide

The 2-methyl group in methyclothiazide should provide information on the effect of the ionization of the exocyclic sulfonamido group alone.

The NMR spectra of methyclothiazide and hydrochlorothiazide were obtained by the Fourier transform technique. This method has been developed for the detection of weak signals from dilute samples and adequate spectra can be obtained for only 10-50 μg of sample. Instead of a continuous signal a pulse mode applies a series of short rf pulses at suitable intervals and one pulse (only a microsecond in duration) encompasses the frequency range in which the particular nuclei absorb energy (95). All of the absorbing nuclei become excited and then return to the ground state, releasing energy which produces the free induction decay (FID). Fourier transformation of the FID yields the frequency spectrum and the repetitive output signals obtained are stored by computer and converted into an NMR spectrum.

The titration of hydrochlorothiazide was performed directly in the NMR tube (96) with 40% NaOD as the concentrated titrant, which minimized dilution (61). All of the chemical shifts are relative to the internal standard, TMS.

The spectrum of each compound was examined before any titrant was added and the resonances were assigned to the protons of the appropriate functional groups, as presented in Table 32. The exchangeable protons are not visible on the NMR spectra since D_2O was part of the solvent system. The spectral assignments did not present any difficulty other than discerning between the aromatic protons at carbons 5 and 8. Theoretically, one would expect electron withdrawing groups to shift the absorption of an aromatic proton to lower field, a deshielding effect, and electron donating groups to shift the absorption to higher field, a shielding effect. Therefore, the aromatic proton at carbon 8 should be at lower field since it is flanked by electron withdrawing sulfonyl groups, while the aromatic proton at carbon 5 should be at higher field since it is flanked by electron donating nitrogen and chlorine atoms. These assignments were, in fact, made by Egan (79) for methyclothiazide and by Boaz (97) for cyclothiazide.

The NMR spectra of methyclothiazide indicate that the aromatic proton resonance at 7.198 ppm shifts significantly to higher field (7.014 ppm) when more than two equivalents of base are added, while the aromatic proton resonance at 8.194 ppm does not exhibit a significant shift under these basic conditions. Similar information for the aromatic protons at carbons 5 and 8 was obtained for hydrochlorothiazide from the titration NMR spectra.

The proton at carbon 8 is adjacent to the exocyclic sulfonamido group and nearby the cyclic sulfonamido group and this proximity should result in a shift of the aromatic proton resonance to higher field. The influence of these ionizing groups on the proton at carbon 5 should be negligible. The NMR spectral evidence obtained by titrations with base led to the assignment of aromatic proton resonances at higher field to carbon 8 and the aromatic proton resonances at lower field to carbon 5, for both methyclothiazide and hydrochlorothiazide. These particular assignments are contrary to those made by Egan (79) and Boaz (97) for the aromatic protons of methyclothiazide and cyclothiazide, respectively.

From the NMR spectra obtained during the titration of hydrochlorothiazide, the chemical shift of the aromatic proton at carbon 8 (H-8) was monitored for the ionization of the exocyclic sulfonamido group while the chemical shift of the methylene protons at carbon 3 (H_2-3) was monitored for the deprotonation of the cyclic sulfonamido group. Due to the structural positions of these representative groups, they were expected to exhibit unique resonances, with respect to the ionization of neighbouring groups. A common resonance involving H-8 was anticipated but the H_2-3 protons were expected to be unaffected by the deprotonation of the exocyclic sulfonamido group. The NMR spectra obtained for methyclothiazide indicated a considerable effect on the methyne proton at carbon 3 as this resonance shifted to higher field (5.441 - 5.367 ppm) when the exocyclic sulfonamido group was titrated. Evidently, both of the representative groups of methyclothiazide exhibit a common resonance and this phenomenon can also be anticipated for hydrochlorothiazide.

The percent of total shift for a particular resonance was determined by dividing the chemical shift of the resonance by its total shift over the entire titration (60). The percentages of the total shift for the two monitored resonances of hydrochlorothiazide at each addition of titrant are presented in Table 34. H_2-3 appears to have shifted more than H-8 after the addition of the first volume of titrant. In theory, the resonance for protons nearest the more acidic group should shift first, but subsequent additions of titrant to the hydrochlorothiazide solution revealed that the two resonances shift more or less simultaneously, since comparable percentages were obtained for each resonance until 2.4 µl of titrant had been added. Approximately 20% of the total shift of H_2-3 occurs when a further 0.1 µl volume of titrant is added, at which point the titration is virtually completed, while H-8 has shifted only 89% after the addition of the same amount of titrant.

The experimental procedure would have to be expanded to include the complete titration of a methyclothiazide sample and, also, the titration of a model compound that is substituted at the exocyclic sulfonamido group is necessary for a complete investigation. The model compound would provide information on the effect of the ionization of the cyclic sulfonamido group on the aromatic proton at carbon 8. The supposedly negligible shifts observed with the titration of the two model compounds could then be subtracted from the chemical shifts, of each site of hydrochlorothiazide, during the titration procedure. Corrections for the effect of one ionization on the shift of protons remote from the site of ionization can then be made, as described by Kesselring and Benet (60). The percentages of ionization calculated

from this data, together with the microconstant equilibria, would represent a much more accurate picture of the order of deprotonation of the acidic hydrogens of the benzothiadiazines.

The information from the preliminary NMR studies suggests that the proton at the 2-position (the cyclic sulfonamido proton) completely dissociates prior to the complete ionization of the exocyclic sulfonamido group. The evidence from the UV absorption spectra of the benzothiadiazines and diazoxide, as well as the effects of the substituents at the 3-position on the pKa values, indicates that this hypothesis is correct.

Table 32. NMR Assignments for Methyclothiazide and Hydrochlorothiazide Before the Addition of Titrant

Assignments for Methyclothiazide	Chemical Shift(ppm)	Multiplicity	Number of Protons
aromatic proton at carbon 5	8.194	singlet	1
aromatic proton at carbon 8	7.198	singlet	1
methyne proton at carbon 3	5.437	triplet	1
methylene protons of chloromethyl group at carbon 3	4.057	doublet	2
methyl protons of 2-methyl group	2.708	singlet	3

Assignments for Hydrochlorothiazide	Chemical Shift(ppm)	Multiplicity	Number of Protons
aromatic proton at carbon 5	8.162	singlet	1
aromatic proton at carbon 8	7.103	singlet	1
methylene protons at carbon 3	4.889	singlet	2

Figure 49. Graphical Representation of Chemical Shift Data for the Titration of Hydrochlorothiazide 0.03 M

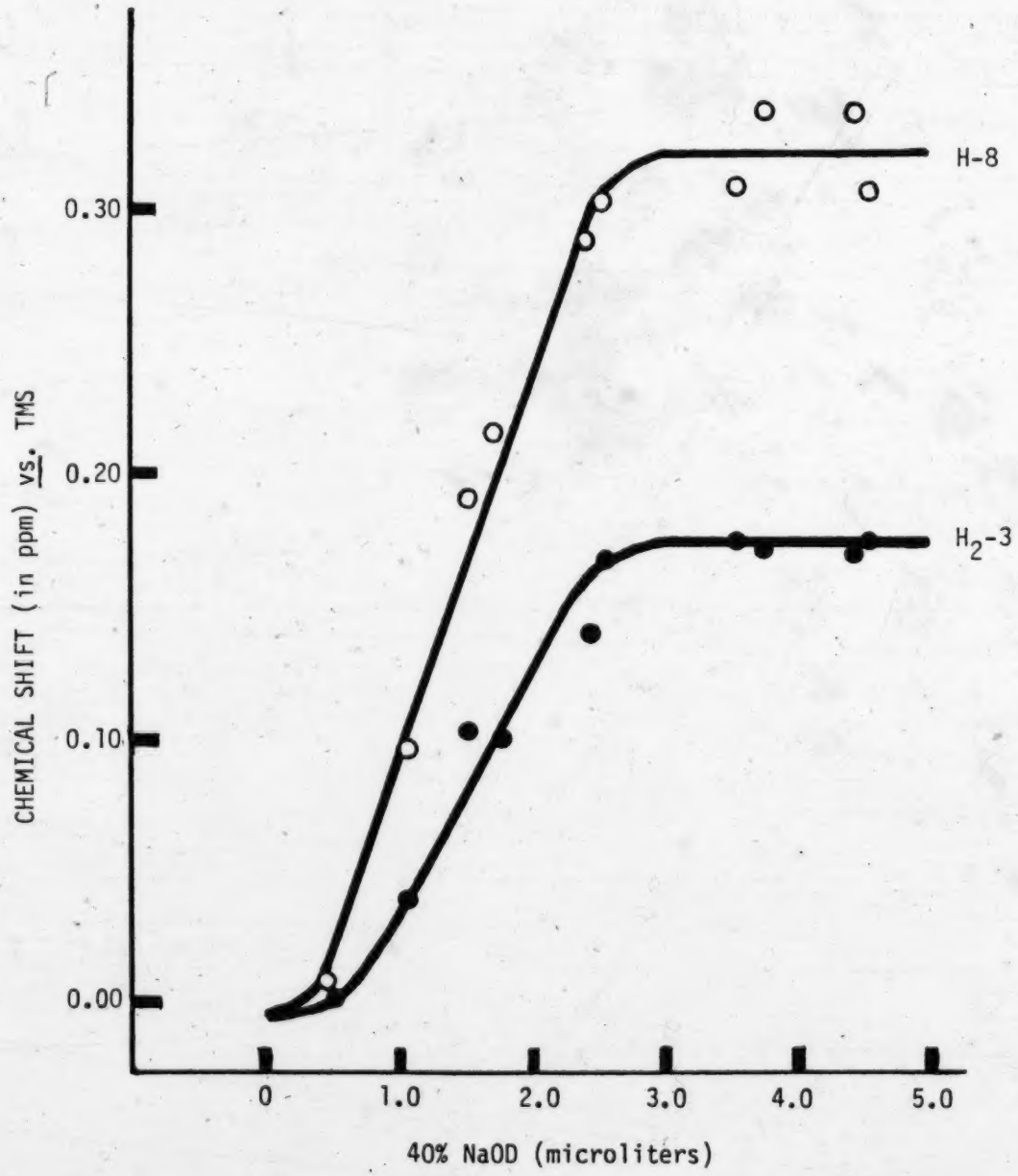


Table 33. Chemical Shift Data for the Titration of Hydrochlorothiazide and Methyclothiazide

Hydrochlorothiazide 0.03 M

40% NaOD (µl)	Chemical Shift of Aromatic Proton at Carbon 8 (ppm)	Chemical Shift of Methylene Protons at Carbon 3 (ppm)
0	7.103	4.889
0.5	7.094	4.883
*1.1	7.010	4.848
1.5	6.914	4.787
*1.7	6.888	4.790
*2.4	6.818	4.752
2.5	6.805	4.721
3.5	6.798	4.713
*3.7	6.767	4.720
*4.4	6.767	4.720
4.5	6.799	4.713

Methyclothiazide 0.02 M

40% NaOD (µl)	Chemical Shift of Aromatic Proton at Carbon 8 (ppm)	Chemical Shift of Methyne Proton at Carbon 3 (ppm)
0	7.198	5.441
2	7.014	5.367

*These volumes of titrant were added to a 0.0225 M solution of hydrochlorothiazide and they have been converted to comparative volumes that would be required for a 0.03 M solution. Details of this conversion are described in the Experimental section.

Table 34. Percent of Total Shift of the Monitored Resonances of Hydrochlorothiazide at Each Incremental Addition of Titrant

Additions of Titrant: 40% NaOD(μl)	Change in Chemical Shift of Aromatic Proton at Carbon 8 (ppm)	% of Total Shift of Aromatic Proton at Carbon 8 (ppm)	Change in Chemical Shift of Methylene Protons at Carbon 3 (ppm)	% of Total Shift of Methylene Protons at Carbon 3 (ppm)
0.5	0.009	2.68	0.006	3.55
1.1	0.093	27.68	0.041	24.26
1.5	0.189	56.25	0.102	60.36
1.7	0.215	63.99	0.099	58.58
2.4	0.285	84.82	0.137	81.07
2.5	0.298	88.69	0.168	99.41
3.7	0.336	100.00	0.169	100.00
4.4	0.336	100.00	0.169	100.00

SUMMARY AND CONCLUSIONS

The attempts to determine the relationship between the ionization properties of the benzothiadiazines and their mechanism of diuretic activity and duration of action have involved the determination of the ionization constants of these drugs.

1. The solubility studies of methyclothiazide and bendroflumethiazide have confirmed decomposition of these agents which occurs under the experimental conditions outlined by Green (70) and used by Ågren and Bäck (18) to determine the pKa_1 value of bendroflumethiazide. These experimental conditions refer specifically to the agitation-equilibration time period in basic buffers and it has been observed that decomposition begins before complete equilibration can be achieved.
2. Although Whitehead et al. (7) and Moskalyk et al. (80) found that the titration of samples with a high organic solvent content did not seriously affect the pKa_1 values of various benzothiadiazines, semi-aqueous titrations would not provide information on the order of deprotonation of the two acidic hydrogens. Also, the validity of the results would be questioned.
3. The UV spectrophotometric method of determining ionization constants realized the determination of thermodynamic values and provided some insight into the order of deprotonation. The highly conjugated ring system of the benzothiadiazines indicates a high molar absorptivity, a property beneficial to obtaining suitable UV absorption spectra. The pKa_1 and pKa_2 values of a number of benzothiadiazines were determined and the pKa_1 values were in good agreement with the literature values, but the difficulty in determining the pKa_2 values is due to the small difference between the molar absorptivities of the

monoprotonated and nonprotonated species. This problem can be alleviated somewhat by the use of buffers of low ionic strength (0.01 M) but the ease of determining the pKa_2 values is not necessarily assured.

4. The pKa_2 values of the benzothiadiazines are probably best determined by the distribution method. A known amount of drug can be partitioned between a suitable water immiscible organic solvent and buffers of low ionic strength. The agitation and equilibration time periods can be controlled and it would be desirable to select an organic solvent that is suitable for all of the compounds under study. This would provide a basis for comparative values. Both the organic and the aqueous phase can be analyzed for drug content by GLC or HPLC and these methods would detect any decomposition that may occur.
5. UV absorption spectra of the thiazides, hydrothiazides, and diazoxide have provided evidence that the more acidic hydrogen is the one at the 2-position. NMR studies have suggested that this hypothesis is correct and that the two ionizations occur almost simultaneously. In addition, NMR spectral evidence has indicated that the 3-subsituent of each of the benzothiadiazines has a considerable influence, not only on the acidity of the proton at the 2-position (7), but also on the acidity of the exocyclic sulfonamido hydrogen. The order of deprotonation can be confirmed by the monitoring of the various chemical shifts during the titration of a model compound which has a substituent in place of one of the exocyclic sulfonamido hydrogens.

Essig (19) found that benzothiadiazines of low pKa_1 are better inhibitors of p-aminohippurate (PAH) transport than are analogs of higher pKa_1. The fact that benzothiadiazines which are appreciably ionized at the pH of the medium are better inhibitors of PAH transport suggests some type of ionic bonding. The ionic bonding to the carrier seems to be an unlikely mechanism of inhibition because the more lipid soluble drugs such as hydrochlorothiazide and cyclopenthiazide (16,17) are more potent diuretics and they have a longer duration of action.

According to Ågren and Bäck (18) hydroflumethiazide and bendroflumethiazide are more protein-bound when ionized and that for bendroflumethiazide a ten-fold increase in ionization results in a three-fold increase in the degree of binding. Hydroflumethiazide binds far less than bendroflumethiazide and these investigators suggest that this is because hydroflumethiazide is less ionized and/or the bulky 3-substituent of bendroflumethiazide contributes to hydrophobic bonding. Evidently, the degree of protein binding influences the renal excretion of hydroflumethiazide and, therefore, the long duration of action of bendroflumethiazide may be due to its high degree of protein binding.

Duggan (17) has proposed a dual mechanism in which both an active transport process and a passive partition process contribute to drug uptake. The active process is of limited capacity and would predominate at low drug concentrations, while the partition process, determined by the polarity of each compound, becomes prevalent at higher drug concentrations.

> "The more polar thiazides would thus resemble PAH, in that the bound pool would be in a facile equilibrium with the mobile one, and metabolic inhibition would result in a rapid net efflux of drug from the tubule. The bound pool of cyclopenthiazide, on the other hand would be maintained." (17)

This suggests that the lipid solubility of the benzothiadiazine determines the extent to which intracellular drug levels are partitioned among the cytoplasm and subcellular organelles.

In any event, the exocyclic sulfonamido group appears to be essential for significant diuretic activity (1,6,9) and the halogen at the 6-position is vital for adequate chloride ion excretion (1,9). There is a definite relationship between the nature of the 3-substituent and diuretic activity (7). Since the exocyclic sulfonamido group is vital for diuretic activity, and this group binds favorably to carbonic anhydrase, the hydrogen at the 2-position would be free to dissociate and, therefore, it would be the more acidic hydrogen.

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APPENDIX

Table 35. Computer Program Reprinted from "The Determination of Ionization Constants" (20)

Line 63 presented an error message and CEPT2=0> was changed to CEPT2=0.0. The computer program was used, with this alteration, in all of the investigations.

Table 4.8 *Computer Programme for calculating overlapping ionization constants using spectrometric data*

```
 1 C      PROGRAM SPECPK
 2 C
 3 C      CALCULATES OVERLAPPING PK VALUES FROM SPECTROSCOPIC
 4 C      DATA
 5 C
 6         COMMON PH(50),D(50),EPS(50),HACT(50),X(50),S(50),
 7        1C(50),A(50),Y(50),NSUBS(40),DENOM(50),FC(50),FM(50),
 8        2FA(50),DELTA1(50),DELTA2(50),CC(50),AA(50),SS(50),
 9        3FACT1(50),FACT2(50),XX(50),YY(50),TK1(50),TK2(50),
10        4PK1(50),PK2(50)
11 C
12 C      READ THE NAME OF THE SUBSTANCE(NSUBS),THE TOTAL
13 C      NUMBER (N) OF READINGS AND THE NUMBER (K) USED FOR
14 C      THE IONIZATION OF THE STRONGER GROUP. READ THE
15 C      CONSTANT VALUES TO BE USED. THESE ARE THE OPTICAL
16 C      DENSITIES OF THE DIPROTONATED SPECIES (DC) AND THE
17 C      NONPROTONATED SPECIES (DA) FOR THE GIVEN CONCENTRATION
18 C      (CONC) AT THE ANALYTICAL WAVELENGTH (WVL). ALLOCATE
19 C      CHARGE TYPE (KTYPE) AS FOLLOWS- ZERO FOR AMPHOLYTE, POSITIVE
20 C      INTEGER FOR DIACIDIC BASES, NEGATIVE INTEGER FOR
21 C      DIBASIC ACIDS TO CALCULATE THE CORRECTIONS FOR THE
22 C      GIVEN IONIC STRENGTH (STREN).
23 C
24    986 READ(3,10)N,K,DC,DA,CONC,KTYPE,WVL,STREN,(NSUBS(I),I=1,40)
25     10 FORMAT(2I5,2F10.3,F14.9,2I4,F10.4,/,40A2)
26        WRITE(4,20) (NSUBS(I),I=1,40)
27     20 FORMAT(1H1,////,30X,12HSUBSTANCE-   ,40A2)
28 C
29 C      READ N VALUES OF PH AND OPTICAL DENSITY(D)
30 C
31        IF (N) 999,999,99
32     99 READ (3,30) (PH(I),D(I),I=1,N)
33     30 FORMAT(2F10.3)
34        EPSC=DC/CONC
35        EPSA=DA/CONC
```

Table 35 (continued)

Table 4.8 ((continued))

```
      FS=STREN**0.5/(1.0+1.5*(STREN**0.5))
      ACT=10.0**(0.509*FS)
      ACT1=10.0**(1.527*FS)
      ACT2=10.0**(2.036*FS)
      DO 1 I=1,N
      FACT1(I)=1.0
      FACT2(I)=1.0
      DELTA1(I)=1.0
      DELTA2(I)=1.0
      EPS(I)=D(I)/CONC
    1 HACT(I)=10.0**(-PH(I))
      WRITE(4,39)
   39 FORMAT (//,28X,17HCONVERGENCE CHECK,//,16X,
     12HK1,11X,2HK2, 9X,7HEPSM(1),3X,7HEPSM(2),
     23X,8HEPSM(AV),1X,1HM,3X,1HJ)
C
C     COMPUTE MOLAR ABSORBANCE OF THE MONOPROTONATED
C     SPECIES (EPSM) USING K RESULTS IN EQUATION (4.7)
C     THIS VALUE WILL BE KNOWN AS CEPT1. IF THE OPTICAL
C     DENSITY OF THE DIPROTONATED SPECIES (DC) IS VERY
C     SMALL OR ZERO, USE (N-K) RESULTS IN EQUATION
C     (4.7A) AS DETAILED BELOW (STATEMENT 106).
C
      AV=0.0
      CK1=0.0
      CK2=0.0
      CEPT1=0.0
      CEPT2=0>
      M=K-1
      J=0
      CHECK=0.05*DA
  156 M=M+1
      J2=N-K
   11 IF(DC.LE.CHECK) GO TO 301
      GO TO 221
  301 IF (J2-J) 252,21,252
  221 IF (M-K) 21,21,252
   21 SUMS=0.0
      SUMC=0.0
      SUMSC=0.0
      SUMS2=0.0
      SUMC2=0.0
      CK1A=CK1
      CK2A=CK2
      DO 2 I=1,M
      SS(I)=(D(I)/CONC)-DELTA1(I)
      S(I)=SS(I)*FACT1(I)
      CC(I)=HACT(I)*(EPSC-SS(I))
      IF (KTYPE) 31,32,33
   31 C(I)=CC(I)/ACT
      GO TO 12
   32 C(I)=CC(I)*ACT
      GO TO 12
```

Table 35 (continued)

Table 4.8 (Continued)

```
 89      33 C(I)=CC(I)*ACT1
 90      12 SUMC=SUMC+C(I)
 91         SUMS=SUMS+S(I)
 92         SUMSC=SUMSC+S(I)*C(I)
 93         SUMS2=SUMS2+S(I)**2
 94       2 SUMC2=SUMC2+C(I)**2
 95         FN=M
 96         DENOM1=(FN*SUMC2-SUMC**2)
 97         SLOPE1=(FN*SUMSC-SUMC*SUMS)/DENOM1
 98         CEPT1=(SUMS*SUMC2-SUMC*SUMSC)/DENOM1
 99         CK=ABS(1.0/SLOPE1)
100         EPSM=CEPT1
101         GO TO 145
102 C
103 C       COMPUTE MOLAR ABSORBANCE (EPSM) USING EQUATION
104 C       (4.7A FOR (N-K) RESULTS. THE VALUE IS KNOWN AS CEPT2.
105 C
106    252 J=N-K
107         M1=K+1
108    253  SUMS=0.0
109         SUMA=0.0
110         SUMSA=0.0
111         SUMS2=0.0
112         SUMA2=0.0
113         CK1A=CK1
114         CK2A=CK2
115         DO4 I=M1,N
116         SS(I)=(D(I)/CONC)-DELTA2(I)
117         S(I)=SS(I)+FACT2(I)
118         AA(I)=(EPSA-SS(I))/HACT(I)
119         IF (KTYPE) 34,35,36
120      34 A(I)=AA(I)*ACT1
121         GO TO 13
122      35 A(I)=AA(I)*ACT
123         GO TO 13
124      36 A(I)=AA(I)/ACT
125      13 SUMA=SUMA+A(I)
126         SUMS=SUMS+S(I)
127         SUMSA=SUMSA+S(I)*A(I)
128         SUMS2=SUMS2+S(I)**2
129       4 SUMA2=SUMA2+A(I)**2
130         FN=J
131         DENOM2=(FN*SUMA2-SUMA**2)
132         SLOPE2=(FN*SUMSA-SUMA*SUMS)/DENOM2
133         CEPT2 =(SUMS*SUMA2-SUMA*SUMSA)/DENOM2
134         CKK=ABS(SLOPE2)
135         EPSM =CEPT2
136         J2=0
137 C
138 C       USE THE MEAN VALUE OF EPSM IN EQUATION (4.5A)
139 C       TO CALCULATE K1 AND K1 USING ALL RESULTS (N).
140 C       THESE VALUES ARE KNOWN AS CK1 AND CK2.
141 C
```

Table 35 (continued)

Table 4.8 (Continued)

```
      145  SUMX=0.0
           SUMX2=0.0
           SUMXY=0.0
           SUMY=0.0
           SUMY2=0.0
           DO 6 I=1,N
           XX(I)=HACT(I)**2*(EPS(I)-EPSC)/(EPS(I)-EPSA)
           YY(I)=-HACT(I)*(EPS(I)-EPSM)/(EPS(I)-EPSA)
           IF(KTYPE)  44,45,46
       44  X(I)=XX(I)/ACT2
           Y(I)=YY(I)/ACT1
           GO TO 14
       45  X(I)=XX(I)
           Y(I)=YY(I)/ACT
           GO TO 14
       46  X(I)=XX(I)*ACT2
           Y(I)=YY(I)*ACT
       14  SUMX=SUMX+X(I)
           SUMY=SUMY+Y(I)
           SUMX2=SUMX2+X(I)**2
           SUMY2=SUMY2+Y(I)**2
        6  SUMXY=SUMXY+X(I)*Y(I)
           FN=N
           DENOM3=(FN*SUMX2-SUMX**2)
           SLOPE=(FN*SUMXY-SUMX*SUMY)/DENOM3
           CEPT=(SUMX2*SUMY-SUMX*SUMXY)/DENOM3
           CK2=ABS(CEPT)
            CK1=1.0/SLOPE
           WRITE(4,40) CK1,CK2,CEPT1,CEPT2,EPSM,M,J
       40  FORMAT( 8X,2E14.5,3F10.2,2I4)
           IF (AV.EQ.EPSM) GO TO 121
C
C          COMPUTE CORRECTION FACTORS TO BE USED IN
C          EQUATIONS (4.7) AND (4.7A). THESE ARE DELTA1 AND
C          AND FACT1, DELTA2 AND FACT2 RESPECTIVELY.
C
           DO8 I=1,N
           FACT1(I)=1.0+CK2/HACT(I)
           FACT2(I)=1.0+HACT(I)/CK1
           DENOM(I)=(HACT(I)**2+CK1*HACT(I)+CK1*CK2)
           FC(I)=HACT(I)**2/DENOM(I)
           FA(I)=CK1*CK2/DENOM(I)
           DELTA1(I)=EPSA*FA(I)
        8  DELTA2(I)=EPSC*FC(I)
C
C          CHECK CONVERGENCE OF SUCCESSIVE VALUES FOR EACH
C          CONSTANT.
C
           EPSILA = ABS(CK1*10.0**(-5))
           EPSILB = ABS(CK2*10.0**(-4))
           DIFFA=ABS(CK1A-CK1)
           DIFFB=ABS(CK2A-CK2)
           IF(DIFFA.GT.EPSILA)GO TO 11
```

Table 35 (continued)

Table 4.8 (Continued)

```
      IF(DIFFB.GT.EPSILB)GO TO 11
      IF (DC.LE.CHECK) GO TO 315
      IF (M-K) 156,166,146
  166 DO 456 I=1,N
      FACT1(I)=1.0
      FACT2(I)=1.0
      DELTA1(I)=0.0
  456 DELTA2(I)=0.0
      GO TO 156
  315 AV=CEPT2
      GO TO 147
  146 AV=(CEPT1+CEPT2)/2.0
  147 EPSM=AV
      GO TO 145
C
C     PREPARE FINAL OUTPUT.
C
  121 WRITE(4,41)(NSUBS(I),I=1,40)
   41 FORMAT (1H1,///,30X,40A2,//,32X,7HRESULTS,//)
      WRITE(4,111) EPSC,CONC,EPSA,MWL,EPSM,STREN
  111 FORMAT(/,10X,31HMOLAR ABSORBANCES-DIPROTONATED=,F8.1,
     112X,14HCONCENTRATION= ,E11.4,/,27X,
     214HNONPROTONATED= ,F8.1,15X,11HWAVELENGTH= ,I4,2HMU,
     3/,26X,15HMONOPROTONATED= ,F8.1,12H(CALC ABOVE),/,61X,
     415HIONIC STRENGTH= ,F6.4,//,11X,2HPH,4X,1HD,7X,3HEPS,
     5 9X,1HX,12X,1HY,11X,3HPK1,4X,3HPK2)
C
C     CALCULATE PK1 AND PK2.
C
      SUM=0.0
      DO18 I=1,K
      TK1(I)=X(I)/(Y(I)-CEPT)
      PK1(I)=ALOG10(1.0/TK1(I))
   18 SUM=SUM+PK1(I)
      FN=K
      AV1=SUM/FN
      WRITE(4,112)(PH(I),D(I),EPS(I),X(I),Y(I),PK1(I),I=1,K)
  112 FORMAT(10X,F4.2,F7.3,F9.1,2E14.5,F9.3)
      SUM=0.0
      K1=K+1
      DO 19 I=K1,N
      TK2(I)=Y(I)-SLOPE*X(I)
      PK2(I)=ALOG10(1.0/TK2(I))
   19 SUM=SUM+PK2(I)
      FN=N-K
      AV2=SUM/FN
      WRITE(4,113)(PH(I),D(I),EPS(I),X(I),Y(I),PK2(I),I=K1,N)
  113 FORMAT(10X,F4.2,F7.3,F9.1,2E14.5,9X,F7.3)
      WRITE(4,132) AV1,AV2
  132 FORMAT(//,64X,12HAVERAGE PK1= ,F5.3,/,
     172X,4HPK2= ,F5.3)
      GO TO 986
  999 CALL EXIT
      END
```

Table 36. Computer Program for the Resolution of Overlapping pKa Values

```
C:FOCAL-11,LFUCA-A

 1.01 E
 1.02 T !!!"*** RESOLUTION OF OVERLAPPING PKA VALUES ***"!!
 1.03 C     FOR 20 DEG C REPLACE D 25 ON LINE 1.8 BY D 26
 1.05 T "INPUT -1 FOR DIBASIC ACIDS,0 FOR AMPHOLYTES,1 FOR"
 1.06 A " DIACIDIC BASES",T,!!
 1.10 A !"ABSORBANCE OF DIPROTONATED SPECIES ",DC
 1.15 A !"ABSORBANCE OF NON-PROTONATED SPECIES ",DA
 1.20 A !"IONIC STRENGTH ",SI,!"PATH LENGTH IN CM ",PL
 1.25 A !"CONCENTRATION IN MOLES PER LITRE ",PM
 1.30 S DC=DC/(PL*PM);S DA=DA/(PL*PM)
 1.35 A !"TOTAL NUMBER OF PAIRS OF DATA ",N
 1.40 A !"NUMBER OF PAIRS OF DATA FOR THE STRONGER SET ",K
 1.45 A !"INPUT DATA IN ORDER OF INCREASING PH"!;S I=0
 1.50 S I=I+1;A !"PH",H(I);S H(I)=FSBR(14,-2.30259*H(I))
 1.55 T "  H+",%2,H(I);A "  ABS",D(I);S D(I)=D(I)/(PL*PM)
 1.60 T "  EPSILON",D(I);A "  OK?",Z;I (FABS(Z)-1.63,1.63
 1.61 S I=I-1;T "XX";G 1.5
 1.63 I (-FABS(I-K))1.65;T !
 1.65 I (I-N)1.5
 1.70 T !!"      K1                    K2            EPSILON M"
 1.75 S SI=FSQT(SI)/(1+1.5*FSQT(SI));S Q=1
 1.80 D 25

 2.10 S SX=0;S SY=0;S SS=0;S SP=0
 2.12 I (.05*DA-DC)2.15;S W=1;G 6.1
 2.15 F I=1,K;D 20
 2.20 S MA=(SY*SS-SX*SP)/(K*SS-SX↑2);S Q=0

 3.10 D 2.1
 3.15 F I=1,N;D 21
 3.20 S KT=(N*SS-SX↑2);S KW=KT/(N*SP-SX*SY)
 3.25 S KT=FABS((SS*SY-SX*SP)/KT)
 3.30 T !%2,KW,"  ",KT,"  ",MA

 4.10 I (.00001-FABS((KW-KA)/KW))4.2
 4.15 I (FABS((KT-KB)/KT)-.0001)5.1
 4.20 S KA=KW;S KB=KT;G 2.1

 5.10 S MB=MA;S KA=KW;S KB=KT;S Q=1;T !

 6.10 D 2.1
 6.15 F I=K+1,N;D 22
 6.20 S MA=(SY*SS-SX*SP)/((N-K)*SS-SX↑2);S Q=0

 7.10 D 3;I (.00001-FABS((KW-KA)/KW))7.2
 7.15 I (FABS((KT-KB)/KT)-.0001)8.05
 7.20 S KA=KW;S KB=KT;G 6.1
```

Table 36 (continued)

```
 8.05 I (W)8.1,8.1;S MB=MA
 8.10 S MA=(MA+MB)/2;T !;D 3;S KT=(SS*SY-SX*SP)/(N*SS-SX↑2);
 8.15 S KW=1/KW;S SQ=0;S SR=0
 8.17 T !!"        H+                  X        Y                    PKA"
 8.20 F I=1,K;D 23
 8.22 T !
 8.25 F I=K+1,N;D 24
 8.30 S SQ=SQ/K;S SR=SR/(N-K)
 8.35 T !!"AVERAGE PKA1",SQ,!"AVERAGE PKA2",SR,!!!!;G

14.10 I (A↑2-.01)14.2;S A=A/2;D 14;S A=A↑2;R
14.20 S A=1+A+A↑2/2+A↑3/6+A↑4/24+A↑5/120+A↑6/720

15.10 I (A↑2-2.04*A+1)15.2;S A=FSQT(A);D 15;S A=2*A;R
15.20 S A=(A-1)/(A+1);S A=2*(A+A↑3/3+A↑5/5+A↑7/7)

20.10 S Y=D(I)-1;S X=H(I)*(DC-D(I)+1);I (-Q)20.2
20.12 S G=KA*KB/(H(I)↑2+KA*H(I)+KA*KB)
20.15 S Y=(1+KB/H(I))*(Y+1-DA*G);S X=(X+H(I)*(DA*G-1))
20.20 I (T)20.25,20.3;S X=X*AW;G 20.35
20.25 S X=X/AA;G 20.35
20.30 S X=X*AA
20.35 S SX=SX+X;S SY=SY+Y;S SS=SS+X↑2;S SP=SP+X*Y

21.10 S X=(H(I)↑2)*(D(I)-DC)/(D(I)-DA)
21.15 S Y=-H(I)*(D(I)-MA)/(D(I)-DA)
21.20 I (T)21.25,21.3;S X=X*AT;S Y=Y*AW;G 21.35
21.25 S X=X/AT;S Y=Y/AW;G 21.35
21.30 S Y=Y/AA
21.35 D 20.35

22.10 S Y=D(I);S X=(DA-D(I))/H(I);I (-Q)22.2
22.12 S G=(H(I)↑2)/(H(I)↑2+KA*H(I)+KA*KB)
22.15 S Y=(1+H(I)/KA)*(Y-DC*G);S X=X+DC*G/H(I)
22.20 I (T)22.25,22.3;S X=X/AA;G 22.35
22.25 S X=X*AW;G 22.35
22.30 S X=X*AA
22.35 D 20.35

23.10 D 21;S Q=FSBR(15,(Y-KT)/X)/2.30259
23.20 T !%,H(I),"  ",X,"  ",Y,"  "
23.30 T Q;S SQ=SQ+Q

24.10 D 21;S R=FSBR(15,1/(Y-KW*X))/2.30259
24.20 D 23.2;T R;S SR=SR+R

25.05 C  FOR 25 DEGREES
25.10 S AA=FSBR(14,SI*1.17892);S AW=FSBR(14,SI*3.53677)
25.20 S AT=FSBR(14,SI*4.71569);R

26.05 C  FOR 20 DEGREES
26.10 S AA=FSBR(14,SI*1.17202);S AW=FSBR(14,SI*3.51605)
26.20 S AT=FSBR(14,SI*4.68806);R
```